New prognostic markers to predict clinical outcome in patients with laryngeal cancer

M.L. Schrijvers

Financial support for printing this thesis was provided by:
ALK-Abello, Atos Medical, Bausch & Lomb, BioMet, Carl Zeiss, Chiesi, DalecoPharma, DosMedical, GlaxoSmithKline, Guide, Hoorgarant, Olympus, MSD, Patiëntenvereniging voor stembandlozen NSvG – Stichting Michel Keijzerfonds, Pentax, Prof. dr. Eelco Huizinga Stichting, Stallergenes, van Lanschot Bankiers

ISBN: 978-90-367-6030-0

Cover:	© Can Stock Photo Inc. / Eraxion
Cover design:	Tom-Paul van der Laan
Printed by:	Proefschriftmaken.nl , Uitgeverij BoxPress
Published by:	Uitgeverij BoxPress, ‘s-Hertogenbosch

rijksuniversiteit groningen

New prognostic markers to predict clinical outcome in patients with laryngeal cancer

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 3 april 2013
om 14.30 uur

door

Michiel Leonard Schrijvers

geboren op 29 juli 1978
te Groningen

Promotores:	Prof. dr. B.F.A.M. van der Laan
	Prof. dr. E. Schuuring
Copromotor:	Dr. J.E. van der Wal
Beoordelingscommissie:	Prof. dr. P.M. Kluin
	Prof. dr. J.A. Langendijk
	Prof. dr. J.L.N. Roodenburg

Paranimfen: Bas Ebels
Elisabeth Schrijvers

Contents

Chapter 1

General introduction

General introduction

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer in the world with an incidence of over 600.000 cases per year and a mortality of 350.000 per year. (http://globocan.iarc.fr/) HNSCC is divided into four different subsites; oral squamous cell carcinoma, pharyngeal squamous cell carcinoma, nasopharyngeal squamous cell carcinoma and laryngeal squamous cell carcinoma (LSCC). Smoking and alcohol consumption are the major risk factors for developing HNSCC, and have a synergistic effect[1,2] . HPV 16 or 18 infection is recognized as a risk factor for developing oropharyngeal carcinoma but in laryngeal cancer HPV related tumours are rarely seen[3]. LSCC accounts for approximately 24% of all new HNSCC with an incidence of 150.677 annually worldwide (129.651 male, 21026 female, http://globocan.iarc.fr/). In the Netherlands, LSCC was diagnosed in 713 patients (602 male, 111 female) in 2008 and 196 patients (154 male, 42 female) died of LSCC in 2008 (http://www.ikcnet.nl). Of all LSCC 67% arise in the glottic, 31% in the supraglottic and 2% in the subglottic region. In the Netherlands all HNSCC are staged by the TNM (Tumor, Node, Metastasis) classification (American Joint Committee on Cancer (AJCC), 7th edition).

An early specific symptom for glottic carcinoma is hoarseness of the voice. Most patients with glottic carcinoma are diagnosed with early stage disease (85% stadium I/II). In contrast, patients with supraglottic carcinoma mostly have a late onset of non specific symptoms such as globus, swallowing disorder and pain. Therefore most patients with supraglottic carcinoma are diagnosed with late stage disease (66% stadium III/IV).

Treatment overview

The optimal treatment for LSCC depends on multiple factors, but is mainly based on tumour location and tumour stage. Besides tumour outcome parameters as

locoregional control, overall survival and disease specific survival several factors are important when choosing the optimal treatment for LSCC. Nowaday, most treatment strategies are based on laryngeal preservation, with preservation of speech and swallowing. For early stage (T1/T2) glottic carcinoma conservative (laser) surgery and radiotherapy (RTH) are both good treatment options with comparable locoregional control, overall survival and disease specific survival[4]. In a selected group of T1a glottic carcinoma with normal or diminished mucosal wave of the vocal cord, CO_2 laser surgery is preferred over RTH because of a better ultimate laryngeal preservation rate[5]. For more advanced stage (T2-T4) glottic carcinoma and supraglottic carcinoma hyperfractionated or accelerated RTH schedules, or combined chemoradiotherapy are good treatment options for laryngeal preservation[6-9]. For advanced stage tumours (T4) with expected low local control rates or worse functional outcome after non-surgical treatment, total laryngectomy with post operative RTH is indicated. Recently, for advance stage tumours (T3/T4), targeted therapy with cetuximab and RTH has been used when chemoradiation is not possible[10,11]. Despite new organ preserving treatment strategies locoregional control and survival has not improved in the past decades. For T1/T2 laryngeal carcinoma, the 5-year local control and overall survival rates vary between 69%-94% (local control) and 63%-82% (overall survival)[12].

Besides the TNM classification few prognostic factors are available to predict clinical outcome after radiotherapy in terms of locoregional control, overall survival or disease specific survival. In the past decades research has concentrated on understanding molecular tumour behaviour and on the identification of new genetic/molecular tumour profiles and individual biomarkers to predict clinical outcome. Theoretically these profiles could be used to predict clinical outcome before treatment, and thereby optimize and personalize the treatment for individual patients. It is difficult to compare different biomarker studies in HNSCC. There are large differences in study populations regarding tumour subsites, TNM stage and treatment modalities. Furthermore, there is no general consensus regarding primary antibodies used, antigen retrieval protocols and scoring methods. This

heterogeneity makes it very difficult to compare different studies and to use molecular profiles to predict clinical outcome in patients. Besides this, for LSCC, only a few good studies finding molecular predictive markers have been published so far. Therefore, in this thesis we studied different biomarkers in a very homogeneous study population consisting of only T1/T2 LSCC all treated with radiotherapy only.

Radiotherapy

Radiotherapy plays an important role in the treatment in the majority of patients with LSCC. The tumour cell DNA is the main target of RTH. Radiotherapy leads to formation of free radicals in cells which will lead to DNA damage. Cell kill after RTH is associated with the level of radiation-induced DNA double strand breaks[13]. There are different mechanisms which lead to cell death after RTH. Cells can undergo apoptosis (programmed cell death), go into senescence (permanent growth arrest) or can undergo mitotic catastrophe[14]. An increasing RTH dose will lead to an increasing amount of tumour cell kill. However, normal tissue will also be damaged during the course of RTH, which can lead to toxic side effects and complications such as dermatisis, mucositis, pain, swallowing dysfunction and xerostomia[15]. To prevent these side effects and allow normal tissue to recover, fractionated RTH is given. Traditionally a small daily dose (1.8-2.0 Gy) was given, daily on weekdays for 7 weeks to achieve a total dosis of 66-70 Gy. Between two radiation sessions, normal tissue can recover and repopulate. However, this also occurs in surviving tumour cells. Several studies show that repopulation of tumour cells limit the effect of RTH and lead to worse locoregional control and survival[16]. To prevent repopulation of tumour cells new fractionated RTH schedules have been developed in the past decades. To reduce the repopulation the same total tumour dose is given in a shorter period. To achieve this, a higher dose per fraction can be given, but this will lead to more late complications[6,17]. Therefore accelerated RTH is given, with a daily dose of 2 Gy, 6 fractions per week. Several studies show a

significant improvement in locoregional control and survival when accelarated RTH is given[6,8,9,18]. Based on these studies, in our institute, most patients with glottic carcinoma T2b-T4 and supraglottic carcinoma T2-T4 are nowadays treated with an accelerated schedule for a total of 66-70 Gy.

Factors influencing radioresistance

Whether a clonogenic tumour cell is killed after ionising radiation depends on multiple factors. Genes/proteins involved in the proliferation rate, cell cycle checkpoint regulation, DNA repair, apoptosis and hypoxia are reported to be associated with reduced response to radiotherapy[19]. In this chapter, the role of hypoxia, FADD and EGFR is discussed in greater detail.

Hypoxia

An important factor influencing tumour radioresistance and thereby worse clinical outcome after RTH is tumour hypoxia. The effect of RTH in tumour cells depends on the rate of oxygenation in the cell. Upon radiation, free radicals are formed which will lead to double-strand DNA breaks and cell death. Oxygen is necessary to stabilize these formed radicals which will lead to more cell kill. Hypoxic cells are 2.5-3 times less radiosensitive than well oxygenated cells[20,21]. It is known that hypoxia occurs in solid tumours. Mainly, two types of hypoxia are described; diffusion limited (chronic) hypoxia and transient (acute or intermittend) hypoxia. In the chronic type hypoxia occurs because of insufficient vascularization in the tumour[22-24]. In transient hypoxia, because of abnormal tumour vascularity, the vessels can be temporarily shut down for a short period of time leaving the surrounding tumour tissue hypoxic for a limited amount of time. It is thought that chronic hypoxia is most responsible for hypoxia-induced radioresistance[20].

There are several ways of measuring hypoxia in a tissue. The classical method is a direct measurement of oxygen in tumour tissue by means of Eppendorf

electrode[25]. This measurement includes values from necrotic tissue. A disadvantage of using this method is that not every type of tumour is easily accessible with an electrode, e.g. tumours in glottic larynx. Another approach to measure hypoxic metabolic active cells is by labeling and subsequently identifying using various methods such as immunohistochemistry or PET scan. Examples of this method are the use of exogenous hypoxic markers such as Pimonidazole[26], or the use of hypoxic tracers like F-MISO PET[27] or 18 FAZA PET[28,29]. A third approach to determine hypoxia is the use of endogenous hypoxic tumour markers such as HIF1α, CA IX, GLUT-1, OPN or LOX detected by immunohistochemistry[30-33]. Of these endogenous markers, HIF1α is studied most intensely. HIF1 is a heterodimeric protein composed of a constitutively expressed HIF1b subunit and an oxygen dependent regulated HIF1α subunit[34,35]. Under normoxia, HIF1α is hydroxylated by prolyl hydroxylase domain 2 (PHD2) on Pro-102 and/or Pro-564. Hydroxylated HIF1α interacts with the von Hippel-Lindau tumour suppressor protein (VHL), which is part of an E3 ubiquitin ligase complex targeting HIF1α for proteasomal degradation[36]. Under hypoxic conditions, hydroxylation is inhibited and thereby HIF1α is stabilized and dimerizes with HIF1b to bind to the hypoxic response element[36] (figure 1). By binding, transcription of target genes is activated. Many of these genes are involved in glucose transporters and glycolysis, survival and proliferation, invasion and metastasis and angiogenesis[34].

In many tumour types hypoxia is associated with worse locoregional control and survival[21,37,38] Possibly, patients with hypoxic tumours may benefit from hypoxic modification. There are different approaches for targeting hypoxia. Several preclinical studies and clinical trials evaluate the effect of agents blocking HIF-1 function, agents attenuating HIF-1 expression, hypoxia-activated bioreductive prodrugs, hypoxia-targeted gene therapy and gene-directed enzyme prodrug therapy[34]. Furthermore studies evaluating the effect of hyperbaric oxygen treatment or the use of hypoxic sensitizers as Nitromidazoles show a good effect on clinical outcome[20]. Such treatment might be a new therapeutic approach to sensitise hypoxic tumours before radiotherapy.

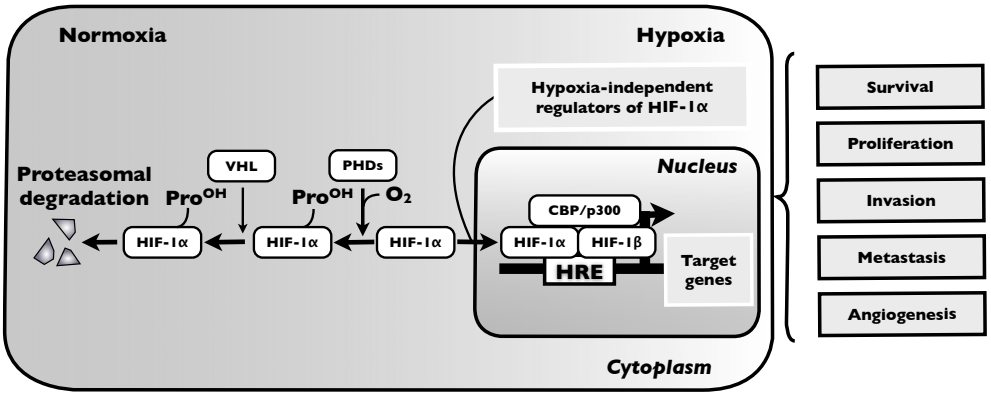

Figure 1. Simplified schematic representation of the HIF1α pathway. Under normoxia, HIF1α is hydroxylated by prolyl hydroxylase domain 2 (PHD2) on Pro-102 and/or Pro-564. Hydroxylated HIF1α interacts with the von Hippel-Lindau tumour suppressor protein (VHL), targeting HIF1α for proteasomal degradation. Under hypoxic conditions, hydroxylation is inhibited and thereby HIF1α is stabilized and dimerizes with HIF1b to bind to the hypoxic response element, thereby activating genes involved in survival, proliferation, invasion, metastasis and angiogenesis. (figure adapted from: Lu X et al. *Clin Cancer Res*. 2010;16(1078-0432; 24):5928-5935.)

11q13 amplification and Fas-associated death domain

Amplification of the 11q13 region is one of the most observed genomic abnormalities in HNSCC and occurs in approximately 30% of all HNSCC tumours[39]. In HNSCC and other tumour types 11q13 amplification is associated with worse overall survival and disease specific survival[40,41]. Gibcus et al. identified 6 genes located in the commonly-amplified chromosome 11q13 region that were amplified and overexpressed in almost all tumours[42]. Of these 6 genes, CCND1 (cyclin D1) and CTTN (cortactin) are studied the most and overexpression of these proteins is associated with worse clinical outcome in different tumour types[43-47]. Gibcus et al. identified Fas (TNFRSF6)-associated death domain (FADD) as the possible driver gene in the 11q13 amplicon responsible for worse disease specific survival and positive lymph node status in advanced oropharyngeal

and laryngeal carcinoma[42] . Originally, FADD was reported as a pro-apoptotic adaptor molecule that recruits caspases 8 and 10 to promote formation of the death-inducing signal complex (DISC)[48,49]. The recruitment of these caspases to the DISC leads to intracellular processing and activation of caspases, eventually resulting in cleavage of downstream targets and apoptosis. More recently, an alternative function for FADD was described as many studies demonstrated that FADD also plays an important role in growth and cell cycle regulation[50,51]. Nuclear localization of FADD has been ascribed to FADD phosphorylation at ser194 (referred to as pFADD) and the highest levels of pFADD are observed at the G2/M phase of the cell cycle[52]. In addition, treatment of cells in vitro with agents blocking the G2/M transition resulted in a significant accumulation of pFADD[53,54]. Finally, expression of a ser194-phospho-mimicking FADD mutant caused G2/M cell cycle arrest[54]. All together these data suggest a key role for FADD/pFADD in cell cycle control. Since cells arrested in the G2/M phase of the cell cycle are most radiosensitive, FADD/pFADD might be a good predictor of clinical outcome in LSCC treated with radiotherapy[55].

The epidermal growth factor receptor (EGFR)

EGFR is a member of the ErbB family of tyrosine kinases, involved in a variety of cellular processes as cell cycle progression or apoptosis. Its structure consists of an extracellular ligand binding domain, a transmembrane region, and an intracellular tyrosine kinase domain with 5 autophosphorylation sites[56,57]. Binding of a ligand for EGFR, such as TGF-1 or EGF, results in auto-phosphorylation of the intracellular tyrosine kinase domain. When activated, the receptors can homodimerise or heterodimerise with HER family members. EGFR activation results in the activation of two major signalling pathways, the Ras/Raf/MEK/ERK pathway and the PI3K/PTEN/AKT pathway, regulating numerous cellular responses, such as increased cell proliferation, decreased insensitivity to apoptosis, migration and differentiation[57-59] (figure 2). Overexpression of EGFR occurs in

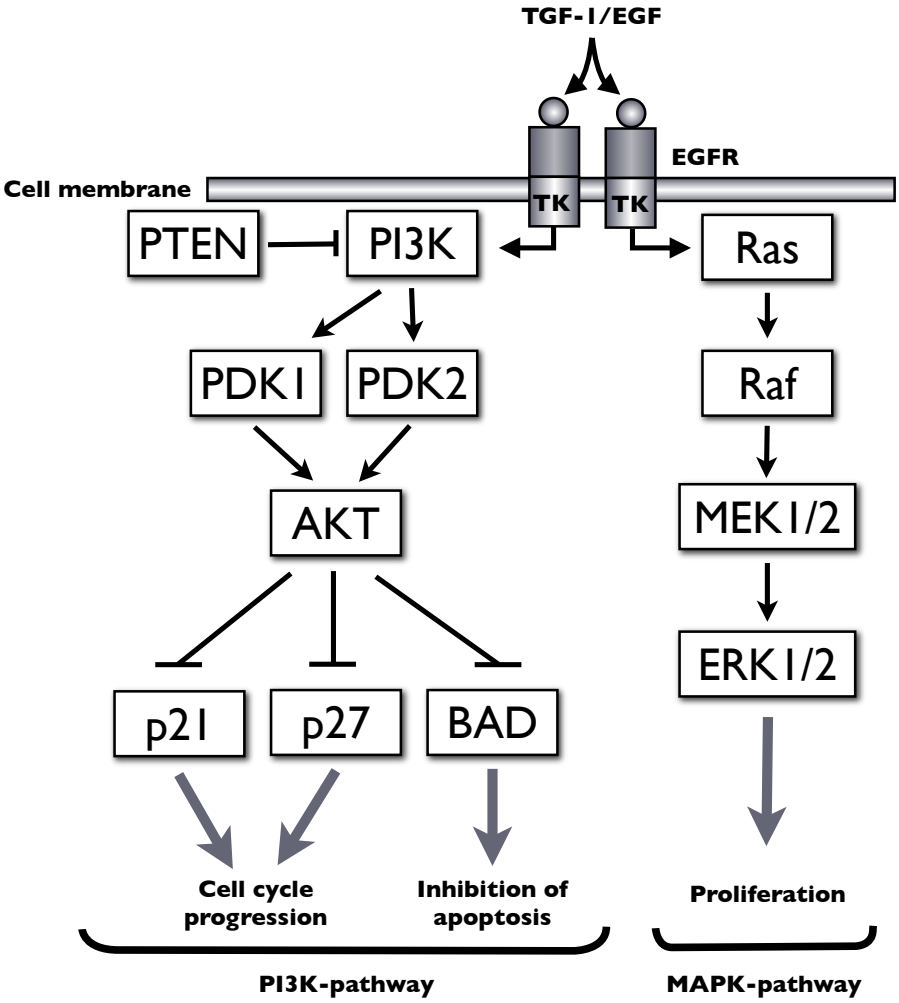

Figure 2. Simplified signaling cascade regulated by EGFR. Binding of a ligand for EGFR, such as TGF-1 or EGF, results in auto-phosphorylation of the intracellular tyrosine kinase domain. When activated, the receptors can homodimerise or heterodimerise with other HER family members. EGFR activation results in the activation of two major signalling pathways, the Ras/Raf/MEK/ERK pathway and the PI3K/PTEN/AKT pathway, regulating numerous cellular responses, such as increased cell proliferation, decreased sensitivity to apoptosis, migration and differentiation. (figure adapted from Rodemann HP *et al. Int J Radiat Biol*. 2007;83(11-12):781-791.)

many different tumour types including HNSCC[56,60,61]. EGFR overexpression is associated with worse clinical outcome in a variety of tumours including lung, bladder, cervix, oesophagus, ovaries, colon and head and neck[62-65]. In early stage LSCC treated with radiotherapy, EGFR overexpression is associated with worse local control and survival[66,67].

Liang et al. reported that EGFR overexpression leads to resistance to radiotherapy, and thus that EGFR is a good candidate for molecular targeting[68]. Nowadays several EGFR blocking agents are commercially available such as cetuximab (Erbitux, IMClone Systems). Cetuximab is a chimeric monoclonal antibody against the ligand-binding domain of EGFR, preventing downstream activation of several targets. In vivo research has shown that blocking of EGFR increases the radiosensitivity of cells[69,70]. In a large clinical trial Bonner et al. reported that for advanced HNSCC concomitant radiotherapy plus cetuximab improved locoregional control compared to radiotherapy alone[10,11]. Since most patients with LSCC are treated with RTH, it would be of great value to establish EGFR as a prognostic marker to predict clinical outcome in early stage LSCC treated with radiotherapy.

The scope of this thesis

Radiotherapy is the preferred treatment modality for stage I-III LSCC because of laryngeal preservation and thereby optimizing quality of life by preservation of speech and swallowing. Radiotherapy can be given in combination with surgery, chemotherapy, receptor blocking agents or as a single treatment modality. For individual patients, it would be useful to predict the tumour response to radiotherapy and the chance of local tumour recurrence and survival. Salvage surgery when radiotherapy fails, is associated with increased complication rates. If a prognostic tumour marker could predict therapy outcome, optimal treatment can be established for individual patients. In HNSCC studies have been performed to identify such a prognostic molecular marker by immunohistochemistry. These

studies however are very heterogeneous regarding antibodies used, antigen retrieval methods and study population. This makes it difficult to compare different results. In this thesis, cell biological markers associated with prognosis and clinical outcome will be investigated in early stage LSCC. For this purpose, we studied a very homogeneous population consisting of patients with mostly early stage (T1/ T2) laryngeal carcinoma treated with RTH only. We constructed a database consisting of patients diagnosed with laryngeal squamous cell carcinoma in the northern part of the Netherlands (comprising >10 medical centers) at the University Medical Center Groningen between 1997-2004. For the more recent studies in this thesis, the database was expanded with patients diagnosed between 2004-2008. In the initial database, 638 patients were included consisting of 433 glottic, 186 supraglottic, 8 subglottic and 11 transglottic tumours. Of the 433 glottic, 360 (83%) were T1/T2. Demographic and clinicopathological data as gender, age, pre-treatment haemoglobin level, T-status, N-status, current and past tobacco use and alcohol use were retrospectively collected by reviewing the patient charts. Of all glottic carcinomas, the pre-treatment paraffin embedded tumour material from 3 large medical centers in the northern part of the Netherlands: the University Medical Center Groningen, the Wilhelmina Hospital Assen and the Scheper Hospital Emmen was collected. The paraffin embedded pre-treatment biopsy material with sufficient carcinoma cells were used for immunohistochemical staining.

Nowadays the treatment for T1a glottic carcinoma is CO_2 laser surgery. The main advantage of using CO_2 laser surgery is that local recurrences can be treated with either a second CO_2 laser surgery or radiotherapy. In case of a local recurrence after primary RTH, salvage laryngectomy is often the only option, with consequently loss of laryngeal function. Up to 1990, RTH was the treatment of choice for T1a glottic carcinoma in our institute. Since then more T1a tumours were treated by CO_2 laser resection. The aim of the study described in **chapter 2** was to compare local control, survival and ultimate laryngeal preservation in patients with T1a glottic carcinoma treated with either CO_2 laser surgery or RTH.

Therefore we performed a retrospective analysis in 100 patients treated with either CO_2 laser surgery or RTH in our institute.

For patients with more advanced tumours of the larynx (T2b glottic and T2 supraglottic or more) treatment with accelerated RTH is the treatment strategy, patients receive 6 fractions of 2 Gy per fraction dose per week for a total dose of 70 Gy. This reduces the total treatment time by 8 days. In the past patients received 5 fractions of 2 Gy per fraction dose for a cumulative total dose of 70 Gy. In **chapter 3** we compared patients treated with conventional radiotherapy to patients treated with accelerated radiotherapy. We studied differences in local control, overall survival and disease specific survival in a retrospective group of 181 patients with glottic or supraglottic carcinoma T2-T4 treated with external beam radiation in our institute. In **chapter 4** we report on validation of FADD and pFADD overexpression on clinical outcome in a homogeneous well defined group of 92 early stage (T1/T2) glottic carcinomas treated with RTH only using immunohistochemistry. In **chapter 5** we studied the role of endogenous hypoxic markers HIF1α, CA-IX and Glut-1 on clinical outcome in the same population described in chapter 4. We also validated a predictive hypoxic profile to choose the optimal treatment (eg hypoxic modification before RTH) in patients with early stage glottic carcinoma. For this purpose immunohistochemistry for HIF1α, CA-IX and Glut-1 was performed on formalin-fixed paraffin-embedded pre-treatment tissue samples of 91 glottic squamous cell carcinomas. Relative tumour staining was scored on the tissue samples and then correlated with clinical data with local control, overall survival and disease specific survival as endpoints. The aim of **chapter 6** was to establish the prognostic effect of epidermal growth factor receptor (EGFR) expression on local control, survival and lymph node metastasis in patients with laryngeal carcinoma treated with radiotherapy only, in a group of 87 glottic and 63 supraglottic T1-T2 LSCC. **Chapter 7** provides a general discussion and conclusion. In **Chapter 8 and 9** an English and Dutch summary of the results is presented.

REFERENCES

1. Freedman ND, Schatzkin A, Leitzmann MF, Hollenbeck AR, Abnet CC. Alcohol and head and neck cancer risk in a prospective study. Br J Cancer. 2007;96(0007-0920; 0007-0920; 9): 1469-1474.
2. Sturgis EM, Wei Q, Spitz MR. Descriptive epidemiology and risk factors for head and neck cancer. *Semin Oncol*. 2004;31(0093-7754; 0093-7754; 6):726-733.
3. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer*. 2011;11(1474-1768; 1474-175; 1):9-22.
4. Hartl DM, Ferlito A, Brasnu DF, et al. Evidence-based review of treatment options for patients with glottic cancer. *Head Neck*. 2011(1097-0347; 1043-3074).
5. Schrijvers ML, van Riel EL, Langendijk JA, et al. Higher laryngeal preservation rate after CO_2 laser surgery compared with radiotherapy in T1a glottic laryngeal carcinoma. *Head Neck*. 2009;31(1097-0347; 1043-3074; 6):759-764.
6. Overgaard J, Mohanti BK, Begum N, et al. Five versus six fractions of radiotherapy per week for squamous-cell carcinoma of the head and neck (IAEA-ACC study): A randomised, multicentre trial. *Lancet Oncol*. 2010;11(1474-5488; 1470-2045; 6):553-560.
7. Bernier J, Domenge C, Ozsahin M, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. *N Engl J Med*. 2004;350(19): 1945-1952.
8. Bourhis J, Overgaard J, Audry H, et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: A meta-analysis. *Lancet*. 2006;368(9538):843-854.
9. Bourhis J, Lapeyre M, Tortochaux J, et al. Phase III randomized trial of very accelerated radiation therapy compared with conventional radiation therapy in squamous cell head and neck cancer: A GORTEC trial. *J Clin Oncol*. 2006;24(1527-7755; 0732-183; 18):2873-2878.
10. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006;354(1533-4406; 6):567-578.
11. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol*. 2010;11(1):21-28.
12. Mendenhall WM, Werning JW, Hinerman RW, Amdur RJ, Villaret DB. Management of T1-T2 glottic carcinomas. *Cancer*. 2004;100(9):1786-1792.

13. Nunez MI, McMillan TJ, Valenzuela MT, Ruiz de Almodovar JM, Pedraza V. Relationship between DNA damage, rejoining and cell killing by radiation in mammalian cells. *Radiother Oncol*. 1996;39(2):155-165.

14. Sato N, Mizumoto K, Nakamura M, et al. A possible role for centrosome overduplication in radiation-induced cell death. *Oncogene*. 2000;19(46):5281-5290.

15. Langendijk JA, Doornaert P, Rietveld DH, Verdonck-de Leeuw IM, Leemans CR, Slotman BJ. A predictive model for swallowing dysfunction after curative radiotherapy in head and neck cancer. *Radiother Oncol*. 2009;90(2):189-195.

16. Kim JJ, Tannock IF. Repopulation of cancer cells during therapy: An important cause of treatment failure. *Nat Rev Cancer*. 2005;5(7):516-525.

17. Bernier J, Bentzen SM. Altered fractionation and combined radio-chemotherapy approaches: Pioneering new opportunities in head and neck oncology. *Eur J Cancer*. 2003;39(0959-8049; 0959-8049; 5):560-571.

18. Overgaard J, Hansen HS, Specht L, et al. Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6 and 7 randomised controlled trial. *Lancet*. 2003;362(1474-547; 9388):933-940.

19. Yeung AH, Sughrue ME, Kane AJ, Tihan T, Cheung SW, Parsa AT. Radiobiology of vestibular schwannomas: Mechanisms of radioresistance and potential targets for therapeutic sensitization. *Neurosurg Focus*. 2009;27(6):E2.

20. Overgaard J. Hypoxic radiosensitization: Adored and ignored. *J Clin Oncol*. 2007;25(1527-7755; 26):4066-4074.

21. Hoogsteen IJ, Marres HA, van der Kogel AJ, Kaanders JH. The hypoxic tumour microenvironment, patient selection and hypoxia-modifying treatments. *Clin Oncol (R Coll Radiol)*. 2007(0936-6555).

22. Overgaard J. Sensitization of hypoxic tumour cells--clinical experience. *Int J Radiat Biol*. 1989;56(0955-3002; 0955-3002; 5):801-811.

23. Overgaard J, Horsman MR. Modification of hypoxia-induced radioresistance in tumors by the use of oxygen and sensitizers. *Semin Radiat Oncol*. 1996;6(1532-9461; 1053-4296; 1):10-21.

24. Moeller BJ, Richardson RA, Dewhirst MW. Hypoxia and radiotherapy: Opportunities for improved outcomes in cancer treatment. *Cancer Metastasis Rev*. 2007;26(0167-7659; 0167-7659; 2):241-248.

25. Gatenby RA, Kessler HB, Rosenblum JS, et al. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int J Radiat Oncol Biol Phys*. 1988;14(0360-3016; 0360-3016; 5):831-838.

26. Nordsmark M, Loncaster J, Aquino-Parsons C, et al. The prognostic value of pimonidazole and tumour pO2 in human cervix carcinomas after radiation therapy: A prospective international multi-center study. *Radiother Oncol*. 2006;80(2):123-131.

27. Rischin D, Hicks RJ, Fisher R, et al. Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: A substudy of trans-tasman radiation oncology group study 98.02. *J Clin Oncol*. 2006;24(1527-7755; 13):2098-2104.

28. Rajendran JG, Schwartz DL, O'Sullivan J, et al. Tumor hypoxia imaging with [F-18] fluoromisonidazole positron emission tomography in head and neck cancer. *Clin Cancer Res*. 2006;12(1078-0432; 18):5435-5441.

29. Grosu AL, Souvatzoglou M, Roper B, et al. Hypoxia imaging with FAZA-PET and theoretical considerations with regard to dose painting for individualization of radiotherapy in patients with head and neck cancer. *Int J Radiat Oncol Biol Phys*. 2007;69(0360-3016; 0360-3016; 2): 541-551.

30. Airley R, Loncaster J, Davidson S, et al. Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. *Clin Cancer Res*. 2001;7(4):928-934.

31. Chan DA, Giaccia AJ. Hypoxia, gene expression, and metastasis. *Cancer Metastasis Rev*. 2007;26(0167-7659; 0167-7659; 2):333-339.

32. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int J Radiat Oncol Biol Phys*. 2002;53(5):1192-1202.

33. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. *Clin Cancer Res*. 2001;7(11):3399-3403.

34. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: Master regulators of metastasis. *Clin Cancer Res*. 2010;16(1078-0432; 1078-0432; 24):5928-5935.

35. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene*. 2010;29(1476-5594; 0950-9232; 5):625-634.

36. Kaelin WG,Jr., Ratcliffe PJ. Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. *Mol Cell*. 2008;30(1097-4164; 1097-2765; 4):393-402.

37. Vordermark D, Brown JM. Endogenous markers of tumor hypoxia predictors of clinical radiation resistance? *Strahlenther Onkol*. 2003;179(0179-7158; 12):801-811.

38. Vaupel P, Mayer A. Hypoxia in cancer: Significance and impact on clinical outcome. *Cancer Metastasis Rev*. 2007(0167-7659).

39. Schuuring E. The involvement of the chromosome 11q13 region in human malignancies: Cyclin D1 and EMS1 are two new candidate oncogenes--a review. *Gene*. 1995;159(0378-1119; 1): 83-96.

40. Akervall JA, Jin Y, Wennerberg JP, et al. Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck. *Cancer*. 1995;76(0008-543; 5):853-859.

41. Meredith SD, Levine PA, Burns JA, et al. Chromosome 11q13 amplification in head and neck squamous cell carcinoma. association with poor prognosis. *Arch Otolaryngol Head Neck Surg*. 1995;121(0886-4470; 7):790-794.

42. Gibcus JH, Menkema L, Mastik MF, et al. Amplicon mapping and expression profiling identify the fas-associated death domain gene as a new driver in the 11q13.3 amplicon in laryngeal/ pharyngeal cancer. *Clin Cancer Res*. 2007;13(1078-0432; 21):6257-6266.

43. Hou H, Chen W, Zhao L, et al. Cortactin is associated with tumour progression and poor prognosis in prostate cancer and SIRT2 other than HADC6 may work as facilitator in situ. *J Clin Pathol*. 2012.

44. Akervall JA, Michalides RJ, Mineta H, et al. Amplification of cyclin D1 in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin D1 overexpression. *Cancer*. 1997;79(0008-543; 2):380-389.

45. Michalides RJ, van Veelen NM, Kristel PM, et al. Overexpression of cyclin D1 indicates a poor prognosis in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg*. 1997;123(0886-4470; 5):497-502.

46. Roy PG, Pratt N, Purdie CA, et al. High CCND1 amplification identifies a group of poor prognosis women with estrogen receptor positive breast cancer. *Int J Cancer*. 2010;127(1097-0215; 0020-7136; 2):355-360.

47. Roy PG, Thompson AM. Cyclin D1 and breast cancer. *Breast*. 2006;15(0960-9776; 0960-9776; 6):718-727.

48. Bhojani MS, Chen G, Ross BD, Beer DG, Rehemtulla A. Nuclear localized phosphorylated FADD induces cell proliferation and is associated with aggressive lung cancer. *Cell Cycle*. 2005;4(11):1478-1481.

49. Lee EW, Kim JH, Ahn YH, et al. Ubiquitination and degradation of the FADD adaptor protein regulate death receptor-mediated apoptosis and necroptosis. *Nat Commun*. 2012;3:978.

50. Hua ZC, Sohn SJ, Kang C, Cado D, Winoto A. A function of fas-associated death domain protein in cell cycle progression localized to a single amino acid at its C-terminal region. *Immunity*. 2003;18(1074-7613; 4):513-521.

51. Osborn SL, Sohn SJ, Winoto A. Constitutive phosphorylation mutation in fas-associated death domain (FADD) results in early cell cycle defects. *J Biol Chem*. 2007;282(0021-9258; 0021-9258; 31):22786-22792.

52. Alappat EC, Feig C, Boyerinas B, et al. Phosphorylation of FADD at serine 194 by CKIalpha regulates its nonapoptotic activities. *Mol Cell*. 2005;19(1097-2765; 3):321-332.

53. Alappat EC, Volkland J, Peter ME. Cell cycle effects by C-FADD depend on its C-terminal phosphorylation site. *J Biol Chem*. 2003;278(0021-9258; 43):41585-41588.

54. Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Konishi N. Phosphorylation status of fas-associated death domain-containing protein (FADD) is associated with prostate cancer progression. *J Pathol*. 2005;206(0022-3417; 4):423-432.

55. Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys*. 2004;59(0360-3016; 4):928-942.

56. Sharafinski ME, Ferris RL, Ferrone S, Grandis JR. Epidermal growth factor receptor targeted therapy of squamous cell carcinoma of the head and neck. *Head Neck*. 2010;32(1097-0347; 1043-3074; 10):1412-1421.

57. Chung KY, Shia J, Kemeny NE, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol*. 2005;23(0732-183; 9):1803-1810.

58. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.

59. Pattje WJ, Schuuring E, Mastik MF, et al. The phosphatase and tensin homologue deleted on chromosome 10 mediates radiosensitivity in head and neck cancer. *Br J Cancer*. 2010;102(1532-1827; 0007-0920; 12):1778-1785.

60. Ozanne B, Richards CS, Hendler F, Burns D, Gusterson B. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. *J Pathol*. 1986;149(0022-3417; 0022-3417; 1):9-14.

61. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res*. 1993;53(0008-5472; 15):3579-3584.

62. Galizia G, Lieto E, Ferraraccio F, et al. Prognostic significance of epidermal growth factor receptor expression in colon cancer patients undergoing curative surgery. *Ann Surg Oncol*. 2006;13(1068-9265; 1068-9265; 6):823-835.

63. Ang KK, Berkey BA, Tu X, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res*. 2002;62(0008-5472; 24):7350-7356.

64. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37 Suppl 4(0959-8049; 0959-8049):S9-15.

65. Pivot X, Magne N, Guardiola E, et al. Prognostic impact of the epidermal growth factor receptor levels for patients with larynx and hypopharynx cancer. *Oral Oncol*. 2005;41(3):320-327.

66. Chang AR, Wu HG, Park CI, Jun YK, Kim CW. Expression of epidermal growth factor receptor and cyclin D1 in pretreatment biopsies as a predictive factor of radiotherapy efficacy in early glottic cancer. *Head Neck*. 2008;30(1097-0347; 1043-3074; 7):852-857.

67. Demiral AN, Sarioglu S, Birlik B, Sen M, Kinay M. Prognostic significance of EGF receptor expression in early glottic cancer. *Auris Nasus Larynx*. 2004;31(0385-8146; 4):417-424.

68. Liang K, Ang KK, Milas L, Hunter N, Fan Z. The epidermal growth factor receptor mediates radioresistance. *Int J Radiat Oncol Biol Phys*. 2003;57(1):246-254.

69. Milas L, Mason K, Hunter N, et al. In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin Cancer Res*. 2000;6(1078-0432; 2):701-708.

70. Nasu S, Ang KK, Fan Z, Milas L. C225 antiepidermal growth factor receptor antibody enhances tumor radiocurability. *Int J Radiat Oncol Biol Phys*. 2001;51(0360-3016; 2):474-477.

Chapter 2

Higher laryngeal preservation rate after CO_2 laser surgery compared to radiotherapy in T1a glottic laryngeal carcinoma

Head Neck. 2009;31(6):759-64

Michiel Schrijvers
Eva van Riel
Johannes Langendijk
Frederik Dikkers
Ed Schuuring
Jacqueline van der Wal
Bernard van der Laan

ABSTRACT

PURPOSE Clinical outcome of endoscopic CO_2 laser surgery and radiotherapy in early stage glottic laryngeal carcinoma is difficult to compare because of differences in treatment selection and patient groups. Therefore we compared local control, overall survival and laryngeal preservation in a homogenous group of patients with T1a glottic carcinoma with normal/diminished mucosal wave treated either with CO_2 laser surgery or radiotherapy.

METHODS AND MATERIALS Retrospective survival analysis was performed on 100 patients with T1a glottic carcinoma treated with CO_2 laser surgery (n=49) or radiotherapy (n=51) diagnosed at the University Medical Center Groningen between 1990-2004.

RESULTS No significant differences in local control and overall survival were found. Ultimate 5-years laryngeal preservation was significantly better in the CO_2 laser surgery group (95% vs. 77%, p=0.043).

CONCLUSIONS Patients with T1a glottic carcinoma with normal/diminished mucosal wave treated with CO_2 laser surgery have a significant better laryngeal preservation rate than patients treated with radiotherapy.

INTRODUCTION

Head and neck cancer is the sixth most common type of cancer in the world with an annual worldwide incidence of 700000 patients[1]. Twenty to thirty percent of these tumours are laryngeal tumours, with the majority arising in the glottis region[2]. Because of the involvement of the vocal folds, most patients with glottic carcinoma present with hoarseness of the voice in an early stage of the disease.
The most commonly used types of treatment for T1a glottic laryngeal carcinoma are radiotherapy and endoscopic CO_2 laser surgery[3]. Clinical outcome in early stage glottic carcinoma (local control, survival and laryngeal preservation) are reported to be similar with both treatment modalities[4-8].

At the University Medical Center Groningen (UMCG), a tertiary referral hospital, CO_2 laser surgery has been increasingly applied as treatment of first choice for T1a glottic laryngeal carcinoma with normal or diminished mucosal wave indicating superficial tumour growth. Radiotherapy used to be the treatment of first choice. Currently, however, radiotherapy is reserved for deeper infiltrating tumours without vocal cord mucosal wave and for recurrences after treatment with CO_2 laser surgery. This shift in treatment of first choice is due to the development of more advanced CO_2 lasers. Currently, no randomized controlled trials comparing radiotherapy and CO_2 laser surgery in early glottic cancer have been performed. Therefore, information regarding the comparison between these two treatment modalities is mainly derived from retrospective studies. In general, the interpretation of these studies is hampered by the fact that in some studies CO_2 laser was preserved for the more superficial tumours, while radiotherapy was applied for the larger, deeper infiltrating tumours[9]. Moreover, in other studies, the selection criteria for choosing between the two treatment options remained unclear[10-12].

Therefore, the main objective of this retrospective analysis was to compare clinical outcome among a well defined subset of patients with T1a glottic laryngeal

carcinoma with normal/diminished mucosal wave treated either with CO_2 laser surgery or radiotherapy.

MATERIAL AND METHODS

Patients

In the period from 1990 to 2004, 242 patients with T1a glottic laryngeal carcinoma were diagnosed at the UMCG. Of these patients, all medical charts were retrospectively revised for clinical data. T1a glottic carcinoma was defined as tumour limited to one vocal cord with normal vocal cord mobility (AJCC cancer staging manual, 6th edition). Videolaryngostroboscopy (VLS) data of 135 patients were available. In 35 patients (26%), mucosal wave of the affected vocal cord was absent, in 85 patients (63%) VLS showed diminished mucosal wave and in15 patients (11%) normal symmetrical vibration patterns of the vocal cords during VLS were reported. All videolaryngostroboscopies were judged by an otorhinolaryngologist in the UMCG at the time of diagnosis. For the purpose of this study, we only included patients with diminished or normal mucosal wave as assessed with VLS. To avoid selection bias, patients with deeper infiltrating tumours were excluded as in these patients radiotherapy was considered standard. Therefore, the final study population was composed of 100 patients. All patients had biopsy proven T1aN0M0 glottic laryngeal carcinoma. Of these 100 patients, 51 were treated with radiotherapy and 49 with CO_2 laser surgery. The choice of treatment was mainly time dependent. Most patients diagnosed before 1997 were treated with radiotherapy, while most patients diagnosed from 1997 were treated with CO_2 laser surgery. Eighty eight patients were male, the median age was 65.5 years and most patients (93%) presented with hoarseness of the voice as primary symptom. Seventy three patients had a smoking history, 10 patients did not smoke and in17 patients this information was not available. Clinical data of patients in both groups is presented in table 1.

Table 1. Patient characteristics: T1a glottic carcinoma diagnosed at the UMCG between 1990-2004

Characteristics	Radiotherapy (n=51)	CO_2 laser surgery (n=49)
Sex		
Male	45 (88%)	43 (88%)
Female	6 (12%)	6 (12%)
Age (years)		
Median (Range)	67 (41-83)	64 (38-83)
Primary symptom		
Hoarse voice	45 (88%)	48 (98%)
Swallowing disorder	0 (0%)	1 (2%)
Other	6 (12%)	0 (0%)
Duration of primary symptom (weeks)		
Median (Range)	20 (0-104)	20 (0-98)
Vocal cord mobility		
Normal	4 (8%)	11 (22%)
Diminished	47 (92%)	38 (78%)
Tobacco use past (per day)		
0	7 (14%)	3 (6%)
1-20	25 (49%)	27 (55%)
>20	10 (20%)	11 (22%)
unknown	9 (18%)	8 (16%)
Tobacco use present (per day)		
0	32 (63%)	31 (63%)
1-20	11 (22%)	10 (20%)
>20	3 (6%)	3 (6%)
unknown	5 (10%)	5 (10%)
Alcohol use past (per day)		
0	12 (24%)	13 (27%)
1-6	27 (53%)	31 (63%)
6	1 (2%)	1 (2%)
unknown	11 (22%)	4 (8%)

This study was approved by the University Medical Center Groningen and written informed consent was given by all patients included in this study.

Treatment

Radiotherapy:

Radiotherapy was delivered using megavoltage equipment, using a 6 MV linear accelerator. All patients were either treated in the UMCG, Isala Clinics Zwolle or the Radiotherapeutic Institute Friesland. The target volume only included the vocal cords and thyroid cartilage. The tumours were irradiated with two opposing lateral fields. All patients were treated with conventional fractionation (2 Gy per fraction, 5 times per week), using a median dose per fraction of 2 Gy (range: 2.0-2.4 Gy) to a median total dose of 66 Gy (range: 60-70 Gy).

CO_2 laser surgery:

Forty nine patients were treated with CO_2 laser surgery, all in the UMCG. Tumour vaporisation was performed endoscopically using a Lumenis laser (Model 30 C, Lumenis Inc, Santa Clara, CA, USA), using the continuous pulse mode. All patients were treated with a type I or II cordectomy according to the ELS criteria[13].

Statistical analysis

Kaplan Meier analysis was performed to measure differences in local control, overall survival and laryngeal preservation between the radiotherapy and CO_2 laser group. Differences were considered to be significant with a p value <0.05 measured by log rank test. To measure differences in clinical patient features, a X^2 test was performed for nominal variables and the student t-test was used for the continuous variables. Local recurrence was defined as tumour recurrence at the primary tumour site, and was calculated from the date of diagnosis until the day of local recurrence or last follow-up. Overall survival was defined as the day of diagnosis until the day of death or last follow-up. All statistical analysis was performed using the statistical package SPSS 14.0.0 (SPSS, inc., Chicago, IL, USA).

RESULTS

Study cohort

No significant differences between the two treatment groups were observed regarding sex, age, primary symptoms, duration of primary symptoms, tobacco use and use of alcohol. However, in the radiotherapy group, the proportion of patients with diminished mucosal wave pattern was significantly higher (X^2 test, p=0.038). However, there were no associations between mucosal wave and the clinical outcome parameters local recurrence, overall survival and laryngeal preservation (data not shown).

No difference in local control and overall survival between the radiotherapy and CO_2 laser group

Of all patients, 25 developed a local recurrence, 12 (24%) in the radiotherapy group and 13 (27%) in the CO_2 laser group. The 5-years local control rate was 73% in the radiotherapy group and 71% in the CO_2 laser group. The difference was not statistically significant (p=0.267, Figure 1a).

In the total population, 13 patients died. Twelve died of disease unrelated to the tumour, and one patient in the radiotherapy group died of disease. Follow up and survival data of both groups are shown in table 2. Kaplan Meier analysis and log rank test showed no statistically significant difference in overall survival between the two groups (p=0.679, Figure 1b).

Laryngeal preservation is better in the CO_2 laser group

In the radiotherapy group, 9 patients with a local recurrence were salvaged with a total laryngectomy, one with CO_2 laser surgery and two patients were given palliative treatment. The patient who was salvaged with CO_2 laser treatment developed a 2nd local recurrence after 2.5 years and ended up with a total laryngectomy as well. In the CO_2 laser group, 9 patients with a local recurrence were treated with radiotherapy, and 4 were given salvage treatment with CO_2 laser

surgery for a second time. In the group of 9 patients treated with salvage radiotherapy, 2 patients developed a 2nd local recurrence, and were then treated with a total laryngectomy. Overall, 12 patients were treated with a total laryngectomy, 10 in the radiotherapy group and 2 in the CO_2 laser group.

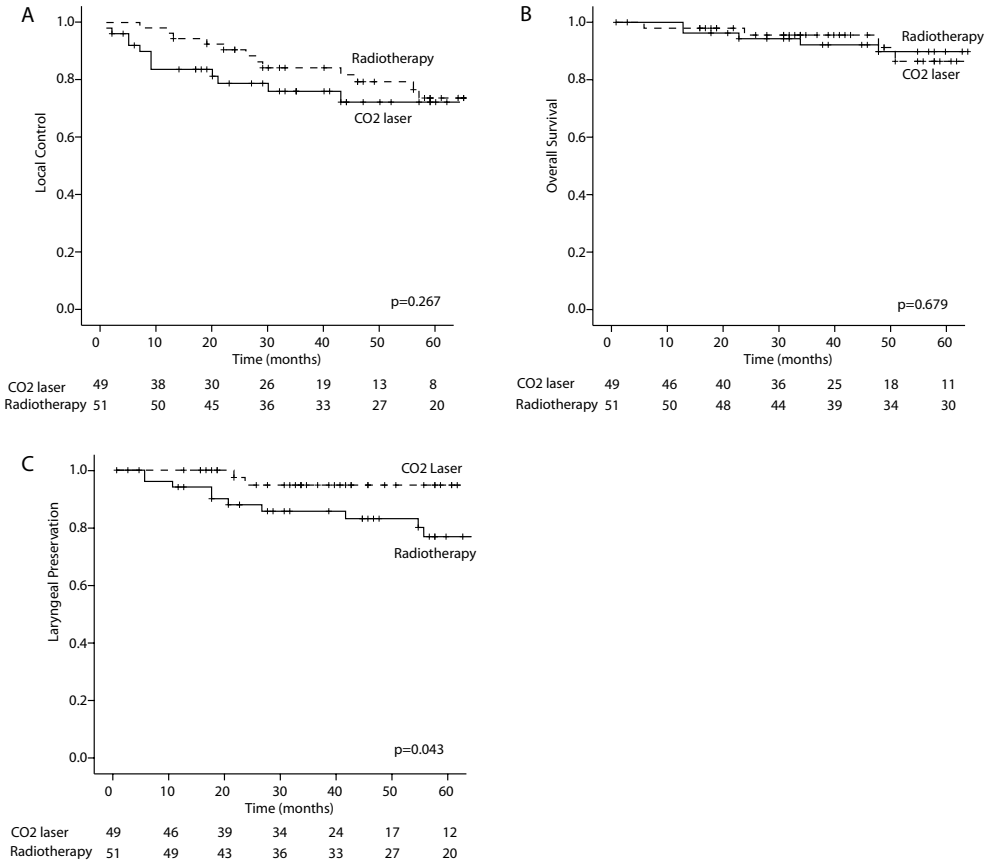

Figure 1. Local control (A), Overall survival (B) and Laryngeal preservation (C) in T1a glottic laryngeal carcinoma treated with primary radiotherapy or CO_2 laser surgery. The 5-year laryngeal preservation rate is significantly better in the CO_2 laser surgery group. P-values are calculated with the log-rank test.

Ultimately, after 5 years, the laryngeal preservation rate in the radiotherapy group was 77% against 95% in the CO_2 laser group (p=0.043, Figure 1c).

Table 2. Patient characteristics: follow-up

Characteristics	Radiotherapy (n=51)	CO_2 laser surgery (n=49)
Events in follow-up		
Local recurrence	12 (24%)	13 (27%)
2nd recurrence	2 (4%)	2 (4%)
2nd primary	9 (18%)	3 (6%)
Death	9 (18%)	4 (8%)
DOD	1 (11%)	0 (0%)
DNOD	8 (89%)	4 (100%)
Treatment local recurrence		
Radiotherapy	0 (0%)	9 (69%)
Total laryngectomy	9 (75%)	0 (0%)
CO_2 laser surgery	1 (8%)	4 (31%)
Palliation	2 (17%)	0 (0%)
Treatment 2nd recurrence		
Total laryngectomy	1 (50%)	2 (100%)
CO_2 laser surgery	0 (0%)	0 (0%)
Palliation	1 (50%)	0 (0%)
Total laryngectomy (total)		
Yes	10 (20%)	2 (4%)
No	41 (80%)	47 (96%)
Median follow-up (months)	64 (12 - 166)	41 (1 - 119)
Time to local recurrence (months)		
Median (Range)	26 (6-56)	8 (1-66)
Time to death (months)		
Median (Range)	48 (13-88)	36 (6-51)

DISCUSSION

CO_2 laser surgery and radiotherapy are both considered effective treatment options in early stage glottic squamous cell carcinoma, with similar reported clinical outcome rates. However, comparison among the different studies reported is difficult because of the differences in selection criteria for either CO_2 laser surgery or radiotherapy as primary treatment. In general, the choice between the two treatment modalities is based on tumour infiltration depth or preference of the physician, but in many studies comparing these modalities not clearly defined[9-12]. Regarding tumour infiltration depth, Goor et al. applied CO_2 laser treatment for the more superficial tumours while radiotherapy was applied for larger, deeper infiltrating tumours[9]. Mortuaire et al. showed that tumour infiltration in the vocal muscle had an adverse effect on local control rate in patients treated with CO_2 laser surgery[14]. This shows that a selection bias may occur when using different criteria to choose between CO_2 laser surgery and radiotherapy. Interpretation is further hampered by differences regarding the distribution of T-classification, which is also a well-known and established prognostic factor for local control. In the last decade, there was a shift from radiotherapy towards CO_2 laser surgery as primary treatment for T1a glottic laryngeal carcinoma. To create a homogenous study group we only included patients with biopsy-proven T1a glottic carcinoma and a normal or diminished mucosal wave measured with VLS. The choice between CO_2 laser or radiotherapy in our group is mostly time dependent, with patients before 1997 treated with radiotherapy, and afterwards with CO_2 laser surgery. Those patients treated with CO_2 laser were all treated with a type I or II cordectomy according to the ELS criteria[13].

In this study we found no statistically significant difference in the 5-years local control rate between the CO_2 laser (71%) and radiotherapy (73%) group. In the literature, local control rates for radiotherapy are reported between 78% and 94%[11,15,16] and for CO_2 laser surgery between 77% and 95%[5,6,9,11,17]. Our 5-years local control rates are somewhat lower than the local control rates found in

literature. A possible explanation might be that other authors reported a 3 year local control rate and included Tis carcinomas in their group[5,6,17].

In the current study, we showed that patients with T1a glottic laryngeal carcinoma treated primarily with CO_2 laser surgery had a significantly better 5-years laryngeal preservation rate (95%) than patients treated with radiotherapy (77%), despite the lack of difference in local control. For the CO_2 laser group, our 5-years laryngeal preservation rate is in concordance with previously published results[11,17]. For radiotherapy, the 5-years laryngeal preservation rates vary between 80% and 95%[4,11]**.** A possible explanation for this variation is the difference in radiotherapy regarding fraction dose, total dose and given schedules.

There are two main advantages of using CO_2 laser surgery as primary treatment modality for T1a glottic laryngeal carcinoma. First, CO_2 laser surgery can be used multiple times in case of a local recurrence. Second, in those cases with a local recurrence in which CO_2 laser surgery is not possible due to tumour expansion, radiotherapy can be administered as effective salvage treatment modality. Our present policy is to use salvage total laryngectomy only in case of tumour recurrence after initial CO_2 laser and salvage radiotherapy treatment.

In the majority of cases with a local recurrence after primary radiotherapy, salvage surgery by total laryngectomy is the only suitable option. Some authors reported on the use of CO_2 laser salvage surgery in case of local recurrence after primary radiotherapy. However, this could only be used for small tumour recurrences with limited tumour spread, as in one of our patients[18,19]. Partial laryngectomy in a previously irradiated area is not considered in most cases, because of the high probability of postoperative complications, such as wound healing problems. Therefore, the majority of patients will be salvaged by total laryngectomy.

In the current analysis, quality of voice was not taken into account. A number of other authors reported on the comparison of the voice quality between the two modalities which, generally, are equal[3,9,10,20]. Jones et al reported a better voice quality after radiotherapy[21]. However, this was tested on a really small study

population consisting of both glottic and supraglottic carcinomas. We did not test the voice quality after treatment, but considering the fact that the ultimate laryngeal preservation rate was significantly better in the CO_2 laser group (95% vs. 77% in the RTH group), the ultimate voice quality will probably be better in the CO_2 laser group.

Some authors reported on cost-effectiveness of CO_2 laser surgery as compared to radiotherapy. Both Goor and Brandenburg showed that CO_2 laser surgery was more cost-effective than radiotherapy[9,22]. However, these results could not be confirmed by others[3,9,22,23]. No studies were found favouring radiotherapy above CO_2 laser surgery when looking at the costs.

CONCLUSION

Both CO_2 laser surgery and radiotherapy are both good treatment options for T1a glottic laryngeal squamous cell carcinoma, with similar local control and survival rates.

Nonetheless, we showed that in a very well defined subset consisting only of T1a glottic laryngeal carcinoma with normal or diminished mucosal wave CO_2 laser surgery is preferred over radiotherapy as the primary treatment because of the better laryngeal preservation rate. This is due to the fact that salvage radiotherapy can be used after primary CO_2 laser therapy for local recurrences, and the ultimate salvage total laryngectomy can be reserved for recurrences after salvage radiotherapy treatment.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005;55(0007-9235; 2):74-108.
2. Hoffman HT, Porter K, Karnell LH, et al. Laryngeal cancer in the united states: Changes in demographics, patterns of care, and survival. *Laryngoscope*. 2006;116(0023-852; 9):1-13.
3. Mendenhall WM, Werning JW, Hinerman RW, Amdur RJ, Villaret DB. Management of T1-T2 glottic carcinomas. *Cancer*. 2004;100(9):1786-1792.
4. Mendenhall WM, Amdur RJ, Morris CG, Hinerman RW. T1-T2N0 squamous cell carcinoma of the glottic larynx treated with radiation therapy. *J Clin Oncol*. 2001;19(0732-183; 20): 4029-4036.
5. Gallo A, de Vincentiis M, Manciocco V, Simonelli M, Fiorella ML, Shah JP. CO_2 laser cordectomy for early-stage glottic carcinoma: A long-term follow-up of 156 cases. *Laryngoscope*. 2002;112(2):370-374.
6. Pradhan SA, Pai PS, Neeli SI, D'Cruz AK. Transoral laser surgery for early glottic cancers. *Arch Otolaryngol Head Neck Surg*. 2003;129(0886-4470; 6):623-625.
7. Rudert HH, Werner JA. Endoscopic resections of glottic and supraglottic carcinomas with the CO_2 laser. *Eur Arch Otorhinolaryngol*. 1995;252(0937-4477; 3):146-148.
8. Warde P, O'Sullivan B, Bristow RG, et al. T1/T2 glottic cancer managed by external beam radiotherapy: The influence of pretreatment hemoglobin on local control. *Int J Radiat Oncol Biol Phys*. 1998;41(0360-3016; 2):347-353.
9. Goor KM, Peeters AJ, Mahieu HF, et al. Cordectomy by CO_2 laser or radiotherapy for small T1a glottic carcinomas: Costs, local control, survival, quality of life, and voice quality. *Head Neck*. 2007;29(1043-3074; 2):128-136.
10. Rosier JF, Gregoire V, Counoy H, et al. Comparison of external radiotherapy, laser microsurgery and partial laryngectomy for the treatment of T1N0M0 glottic carcinomas: A retrospective evaluation. *Radiother Oncol*. 1998;48(0167-8140; 2):175-183.
11. Spector JG, Sessions DG, Chao KS, et al. Stage I (T1 N0 M0) squamous cell carcinoma of the laryngeal glottis: Therapeutic results and voice preservation. *Head Neck*. 1999;21(1043-3074; 8): 707-717.

12. Thurnher D, Erovic BM, Frommlet F, et al. Challenging a dogma - surgery yields superior long-term results for T1a squamous cell carcinoma of the glottic larynx compared to radiotherapy. *Eur J Surg Oncol*. 2007(0748-7983).

13. Remacle M, Van HC, Eckel H, et al. Proposal for revision of the european laryngological society classification of endoscopic cordectomies. *Eur Arch Otorhinolaryngol*. 2007;264(0937-4477; 5): 499-504.

14. Mortuaire G, Francois J, Wiel E, Chevalier D. Local recurrence after CO_2 laser cordectomy for early glottic carcinoma. *Laryngoscope*. 2006;116(1):101-105.

15. Reddy SP, Hong RL, Nagda S, Emami B. Effect of tumor bulk on local control and survival of patients with T1 glottic cancer: A 30-year experience. *Int J Radiat Oncol Biol Phys*. 2007;69(0360-3016; 5):1389-1394.

16. Lee JH, Machtay M, McKenna MG, et al. Radiotherapy with 6-megavolt photons for early glottic carcinoma: Potential impact of extension to the posterior vocal cord. *Am J Otolaryngol*. 2001;22(0196-0709; 1):43-54.

17. Hartl DM, de ME, Hans S, Janot F, Brasnu D. Treatment of early-stage glottic cancer by transoral laser resection. *Ann Otol Rhinol Laryngol*. 2007;116(0003-4894; 11):832-836.

18. Ansarin M, Planicka M, Rotundo S, et al. Endoscopic carbon dioxide laser surgery for glottic cancer recurrence after radiotherapy: Oncological results. *Arch Otolaryngol Head Neck Surg*. 2007;133(0886-4470; 12):1193-1197.

19. de Gier HH, Knegt PP, de Boer MF, Meeuwis CA, van d,V, Kerrebijn JD. CO_2-laser treatment of recurrent glottic carcinoma. *Head Neck*. 2001;23(1043-3074; 3):177-180.

20. Peeters AJ, van Gogh CD, Goor KM, Verdonck-de L,I, Langendijk JA, Mahieu HF. Health status and voice outcome after treatment for T1a glottic carcinoma. *Eur Arch Otorhinolaryngol*. 2004;261(0937-4477; 10):534-540.

21. Jones AS, Fish B, Fenton JE, Husband DJ. The treatment of early laryngeal cancers (T1-T2 N0): Surgery or irradiation? *Head Neck*. 2004;26(1043-3074; 2):127-135.

22. Brandenburg JH. Laser cordotomy versus radiotherapy: An objective cost analysis. *Ann Otol Rhinol Laryngol*. 2001;110(0003-4894; 4):312-318.

23. Gregoire V, Hamoir M, Rosier JF, et al. Cost-minimization analysis of treatment options for T1N0 glottic squamous cell carcinoma: Comparison between external radiotherapy, laser microsurgery and partial laryngectomy. *Radiother Oncol*. 1999;53(0167-8140; 1):1-13.

Chapter 3

Conventional versus accelerated radiotherapy in T2-T4 laryngeal squamous cell carcinoma

Submitted

Michiel Schrijvers
Jasper Wachters
Jacqueline van der Wal
Ed Schuuring
Bernard van der Laan
Johannes Langendijk

ABSTRACT

PURPOSE To evaluate whether the clinical introduction of accelerated radiation therapy (ART) in T2-T4 laryngeal carcinoma improved outcome.

METHODS AND MATERIALS In this retrospective study, 181 patients with T2b-T4 glottic or T2-T4 supraglottic laryngeal carcinoma primarily treated with conventional fractionation (CF), or accelerated radiotherapy (ART) who were treated at the University Medical Center Groningen (UMCG) were included. Univariate and multivariate analysis was performed to compare the two fractionation schedules in a single centre setting with regard to local control (LC), overall survival (OS) and disease specific survival (DSS).

RESULTS In the multivariate analysis, LC was significantly better in patients treated with ART than those treated with CF (HR 1.76, 95% CI 1.01-3.05). Other independent prognostic factors associated with better LC were N0-status (HR 2.13, 95% CI 1.14-3.98) and female sex (HR 2.69, 95% CI 1.25-5.76). Improved LC did not translate into a significant improvement of the OS or DSS. Supraglottic tumour location (HR 1.67, 95% CI 1.04-2.69) and age $\geq$ 64 years (HR 2.02, 95% CI 1.35-3.05) were both associated with worse OS. Positive N-status (HR 3.88, 95 % CI 1.57-9.60), male sex (HR 7.30, 95% CI 2.02-26.37) and age <64 (HR 2.76, 95% CI 1.27-6.02) were predictors of worse DSS in multivariate analysis.

CONCLUSION In T2-T4 laryngeal carcinoma, ART significantly improved LC compared to CF, but this improvement did not translate into a significant improvement of either OS or DSS.

INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) represents the largest group among head and neck squamous cell carcinoma (HNSCC) with 550.000 cases per year worldwide[1]. For LSCC, a variety of primary treatment options are available, including surgery, radiation therapy or both either or not combined with chemotherapy[2-4]. In many cases, the primary treatment consists of radiotherapy with or without neoadjuvant or concurrent chemotherapy in order to preserve the larynx, while primary surgery is applied in selected cases in which non-surgical treatment is expected to result in low local control rates and/or worse functional outcome[5].

In the past, radiotherapy was applied using standard fractionation, including 2 Gy per fraction to a total dose of 66 Gy to 70 Gy, 5 times per week, over a period of 7 weeks. In the last decades, numerous studies reported on the additional value of altered fractionation schedules, including accelerated radiotherapy with or without total dose reduction and/or hyperfractionated radiotherapy.

In the early 2000's, two randomised controlled trials showed a significant improvement in locoregional control among patients treated with either hyperfractionated or ART compared to CF[6,7] (DAHANCA 6&7 and RTOG). The DAHANCA trial showed a significant effect on local tumour control in favour of the ART group compared to the CF group (76% vs. 64% after 5 years). The RTOG trial also showed a significant improvement of locoregional control among patients treated with either hyperfractionated or ART compared with CF (54.4% vs. 54.5% vs. 46% after 2 years respectively). In these studies, no significant association was found between fractionation schedule and OS.

Until 2000, at the department of Radiation Oncology of the University Medical Center Groningen (UMCG), patients with T2-T4 laryngeal carcinoma were treated with conventional fractionated radiotherapy. After the publication of a number of randomized controlled trials, accelerated radiotherapy was increasingly used as the new standard in case chemoradiation was considered not feasible.

It is not always obvious whether the beneficial effects as reported in randomised controlled trials and/or meta-analysis always translate into improved outcome in single institutions. From this point of view, we wondered if we could demonstrate a beneficial effect of the clinical introduction of accelerated radiotherapy without total dose reduction instead of standard fractionation in our own institution.

Therefore, the purpose of this retrospective study was to test the hypothesis that the clinical introduction of accelerated RT indeed resulted in better local control compared to conventional RT in T2-T4 LSCC.

MATERIAL AND METHODS

Patients

Between 1986 and 2006, 583 patients were treated for LSCC at our hospital. To be included in this study, patients had to fulfil the following eligibility criteria: (1) histologically proven squamous cell carcinoma; (2) located in the glottic or supraglottic region; (3) cT2, cT3 or cT4 supraglottic or cT2b, cT3 or cT4 glottic; (4) curatively treated with radiotherapy alone; (5) no other previous treatment, and (6) treated at the UMCG. Eventually, the study population was composed of 181 patients that fulfilled all eligibility criteria. Of these 181 patients, 117 had T2 (65%), 44 T3 (24%) and 20 T4 tumours (11%). Most patients were excluded because of non eligible T-status, including 210 patients with T1 and 74 patients with T2a disease. In addition, 114 patients were treated with a combination of surgery and radiotherapy and were therefore not included. The patient and tumour characteristics of patients included in this study are listed in Table 1.

Radiotherapy

Radiotherapy was delivered using megavoltage equipment (6 MV linear accelerator). Patients treated before 1998 were treated with 2D conventional radiotherapy based on direct simulation. In patients treated from 1998, a planning CT scan was made in supine position. Patients were treated with fraction doses of

Table 1. Patient characteristics and tumour data per treatment group

Characteristics	6 fractions per week (N=83)	5 fractions per week (N=98)	Total No. (%) N=181
Sex			
Male	61 (74%)	79 (81%)	140 (77%)
Female	22 (26%)	19 (19%)	41 (23%)
Age (years)			
Median (Range)	64 (35-83)	64 (33-84)	64 (33-84)
Primary symptom			
Hoarse voice	53 (64%)	77 (79%)	130 (72%)
Swallowing disorder	10 (12%)	2 (2%)	12 (6%)
Globus	3 (3%)	2 (2%)	5 (3%)
Pain	8 (10%)	13 (13%)	21 (12%)
Referred pain	4 (5%)	0 (0%)	4 (2%)
Other	5 (6%)	4 (4%)	9 (5%)
T-status			
T1	0 (0%)	0 (0%)	0 (0%)
T2	37 (45%)	80 (82%)	117 (65%)
T3	36 (43%)	8 (8%)	44 (24%)
T4	10 (12%)	10 (10%)	20 (11%)
N-status			
N0	52 (63%)	79 (81%)	132 (73%)
N+	30 (37%)	19 (19%)	49 (27%)
Site			
Glottis	26 (31%)	49 (50%)	75 (41%)
Supraglottis	57 (69%)	49 (50%)	106 (59%)

2.0 Gy up to a median total dose of 70 Gy, 5 times per week in 7 weeks (conventional fractionation) or 6 times per week with a fraction dose of 2.0 Gy (accelerated fractionation) to the same total dose. In case of the ART, a concomitant boost technique was used. These patients were generally treated with a second fraction on Friday afternoon with a minimum interval of 6 hours between fractions. Most patients received bilateral elective irradiation of the neck nodes to a total dose of 46 Gy and a boost on the primary tumour and pathological lymph nodes to a total dose of 70 Gy.

Eventually, 83 patients (46%) were treated with ART with a median overall treatment time (OTT) of radiation of 39 days (range: 32-41 days), while 98 patients were treated with conventional fractionation with a median OTT of 50 days (range 44-74 days).

Follow up data

After completing radiation, patients were followed every 3 months during the first and second year, and every 6 months during the third, fourth and fifth year. After five years without evidence of disease, patients were discharged from further follow-up.

Statistical analysis

Differences in pre-treatment characteristics between the accelerated and conventionally treated group were tested with the Pearson chi-square test.

Follow-up time was calculated from the day of diagnosis until the date of the last follow-up visit. Local recurrence was defined as tumour recurrence at the primary tumour site and was calculated from the date of diagnosis until the day of local recurrence diagnosis or to the last follow-up. Overall survival (OS) was defined from the day of diagnosis to the date of death or to last follow-up.

In the univariate analysis, local control (LC), disease specific survival (DSS) and OS were estimated with the Kaplan-Meier method. To test the statistical significance of differences between curves, the log–rank test was used. A multivariate analysis using the Cox proportional hazards model was performed to identify covariates that were significantly associated with these endpoints. The following factors were entered into the multivariate model: T classification (T1-T2 vs. T3-T4), N classification (N0 vs. N+), tumour site (glottic vs. supraglottic), RT schedule (ART vs. CF), sex (male vs. female) and age (<64 years vs. >64).

All statistical analysis was performed using the Statistical Package for Social Sciences, version 16.0 (SPSS, Chicago, IL).

RESULTS

The median follow up time was 42 months. Seventy patients (39%) developed a local recurrence, of which 58 (83%) were salvaged by a total laryngectomy, one was treated with CO_2 laser surgery and 11 patients received palliative treatment because local recurrences were considered unresectable. Seven patients developed a second local recurrence after salvage total laryngectomy. Six out of these 7 patients received palliative therapy and one patient was successfully re-salvaged with a total pharyngectomy with postoperative re-irradiation. Three patients developed a regional recurrence after salvage total laryngectomy, which all received palliative therapy. Four patients developed a regional recurrence of which 2 were treated with salvage neck dissections and 2 received palliative therapy because recurrence was considered unresectable. Of all patients, seven patients developed distant metastases, which were treated with palliative therapy. One hundred patients (55%) died during follow up. Of these patients 30 (30%) died of laryngeal cancer, and 65% died of non tumour related disease.

Local Control

In the univariate analysis, the 5-year local control rate among patients treated with ART was 68% which was significantly better than that observed among those treated with CF in which the 5-year local control rate was only 48% (hazard ratio (HR) 2.00, 95% confidence interval (CI) 1.21-3.30, p=0.005, Figure 1). In addition, cT3-T4 status, female sex and supraglottic tumour site were associated with significantly better LC rates (Table 2). No association was found with pre-treatment nodal status or age.

In the multivariate analysis, CF (HR 1.76, 95% CI 1.01-3.05), male sex (HR 2.69, 95% CI 1.25-5.76) and positive N-status (HR 2.13, 95% CI 1.14-3.98) were independent adverse prognostic factors for local control (Table 4).

Table 2. Results of the univariate analysis regarding local control

Characteristics	No of patients (%)	LC (%)	Log-rank P value	HR (95% CI)
Tumour site				
Glottic	75 (41)	51	**0.018**	**1.74 (1.09-2.78)**
Supraglottic	106 (59)	69		**1**
T-status				
T2	117 (65)	53	**0.004**	**2.23 (1.26-3.95)**
T3-4	66 (35)	77		**1**
N-status				
N-	132 (73)	62	0.654	1
N+	49 (27)	60		1.12 (0.67-1.89)
RTH Schedule				
5 fractions p/w	98 (54)	52	**0.005**	**2.00 (1.21-3.30)**
6 fractions p/w	83 (46)	72		**1**
Gender				
Male	140 (77)	55	**0.002**	**2.97 (1.42-6.21)**
Female	41 (23)	80		**1**
Age				
<64	97 (54)	55	0.29	1.29 (0.79-2.10)
>64	84 (46)	68		1

LC: local control; OS: overall survival, HR: hazard ratio; CI: confidence interval

Regional control

In the univariate analysis, no significant association was found between patients treated with ART or CF and regional control (HR 3.60, 95% CI 0.38-34.63).

Survival

In the univariate analysis, OS was significantly associated with N-classification and primary tumour site. No significant association was found for RT regimen (Table 3). In the multivariate analysis, age ≥64 years (HR 2.02, 95% CI 1.35-3.05) and supraglottic tumour site (HR 1.67, 95% CI 1.04-2.69) were independent adverse prognostic factors for OS. Pre-treatment positive N-status (HR 1.58, 95%

CI 0.96-2.61) showed a trend towards significance, but did not reach significant level.

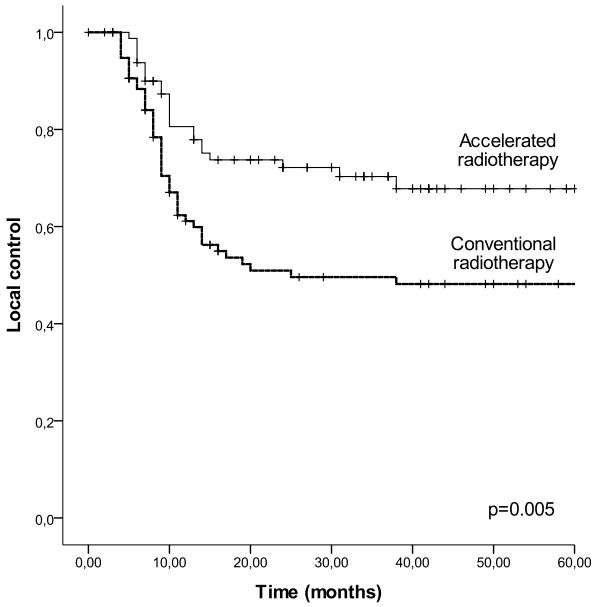

Figure 1. Local control (LC) rate as a function of RT schedule.

Disease specific survival

In the univariate analysis, the DSS was significantly worse after ART. In addition, advanced T-stage, N+ status, and gender were significantly associated with DSS (table 3). In the multivariate analysis, pre-treatment positive N-status (HR 3.88, 95% CI 1.57-9.60), male sex (HR 7.30, 95% CI 2.02-26.37) and age <64 (HR 2.76, 95% CI 1.27-6.02) were independent adverse prognostic factors for DSS (Table 4).

Table 3: Results of the univariate analysis regarding OS and DSS

Characteristics	No of patients (%)	OS (%)	Log-rank P value	HR (95% CI)	DSS (%)	Log-rank P value	HR (95% CI)
Tumour site							
Glottic	75 (41)	51	**0.006**	**1**	68	0.761	1.12 (0.53-2.38)
Supraglottic	106 (59)	41		**1.77 (1.17-2.69)**	71		1
T-status							
T2	117 (65)	41	0.147	1.38 (0.89-2.16)	75	**0.007**	**1**
T3-4	66 (35)	52		1	58		**2.64 (1.26-5.50)**
N-status							
N0	132 (73)	49	**0.003**	**1**	79	**<0.001**	**1**
N+	49 (27)	36		**1.88 (1.23-2.88)**	50		**3.62 (1.73-7.5)**
RTH Schedule							
5 fractions p/w	98 (54)	33	0.433	1.20 (0.76-1.90)	77	**0.001**	**1**
6 fractions p/w	83 (46)	59		1	56		**3.32 (1.56-7.05)**
Sex							
Male	140 (77)	45	0.950	1	65	**0.034**	**3.37 (1.02-11.15)**
Female	41 (23)	44		1.02 (0.63-1.63)	87		**1**
Age							
<64	97	52	0.058	1	46	0.065	1.85 (0.88-3.90)
>64	84	37		1.46 (0.98-2.17)	53		1

OS: overall survival; HR: hazard ratio; CI: confidence interval; DSS: disease specific survival

Table 4. Multivariate analysis regarding LR, OS and DSS

Characteristics	Hazard ratio LR (95% CI)	Hazard ratio OS (95% CI)	Hazard ratio DSS (95% CI)
Tumour site			
Glottic	1	**1**	1.54 (0.68-3.50)
Supraglottic	1.50 (0.88-2.57)	**1.67 (1.04-2.69)**	1
T-status			
T2	1	1	1
T3-4	1.83 (0.96-3.48)	1.04 (0.62-1.73)	2.06 (0.81-5.19)
N-status			
N-	**1**	1	**1**
N+	**2.13 (1.14-3.98)**	1.58 (0.96-2.61)	**3.88 (1.57-9.60)**
RTH Schedule			
5 fractions p/w	**1.76 (1.01-3.05)**	1.05 (0.64-1.72)	1
6 fractions p/w	**1**	1	2.29 (0.98-5.37)
Sex			
Male	**2.69 (1.25-5.76)**	1.17 (0.72-1.92)	**7.30 (2.02-26.37)**
Female	**1**	1	**1**
Age			
<64	1.28 (0.79-2.08)	**1**	**2.76 (1.27-6.02)**
>64	1	**2.02 (1.35-3.05)**	**1**

LR: local recurrence; DSS: disease specific survival; CI: confidence interval

DISCUSSION

Since 2000, in our institution most patients with glottic carcinoma T2b-T4 and supraglottic carcinoma T2-4 have been treated with ART. This retrospective study confirmed that patients treated with ART showed significantly better local control rate than patients treated with CF. However, this significant improvement in LC did not translate into a significant benefit in terms of OS and DSS.

More recently, the MARCH meta-analysis showed a significant OS benefit among patients treated with altered fractionated radiotherapy in HNSCC[1]. This

meta-analysis included tumours of the oral cavity, oropharynx, larynx and hypopharynx. The positive effect on OS was mainly seen in the patients treated with hyperfractionated radiotherapy, with an absolute difference of 8% after 5 years, while the absolute benefit among patients treated with accelerated radiotherapy was much lower, i.e., 2% after 5 year. In the same meta-analysis, altered fractionation was associated with a significant improvement of locoregional control compared to CF group (6.4% after 5 years). This effect was mainly seen after hyperfractionated RT (9.4%) and ART group without total dose reduction (7.3%), and was mainly due to better local control, while no significant effect was noted for nodal control. The youngest patient group (50 or less) had the most benefit from ART with better overall survival, disease specific survival and locoregional control rates.

The results found in the current retrospective study are in line with the results found in the meta-analysis, indicating that the clinical introduction of accelerated radiotherapy into routine clinical practice indeed resulted in a similar improvement of LC as observed in earlier prospective randomized studies.

In the current analysis, no significant effect was found of ART on OS. A possible explanation for the lack of effect of ART on overall survival is the high incidence of non-tumour related deaths in our population (65%). Since most patients in our study had a history of tobacco and alcohol abuse (results not shown), the risk of dying of co-morbid heart diseases and/or other tumour types is increased in this population. In our study, supraglottic tumor location and age >64 were independent predictors of worse OS. The OS among elderly patients was significantly worse as compared to the younger age group, which could possibly be explained by higher rates of co-morbidity among the elderly patients. However, this was not evaluated in the current study. Our results are however in line with two previous reports in which age was identified as unfavorable prognostic factor.[8,9]. A striking finding was that the younger patients had significantly worse DSS. There are two possible explanations for these findings. First, the higher rate of non-cancer related death in the elderly patients might be a competitive risk for developing

locoregional tumour recurrence or distant metastases. Second, the biological behavior in the younger patient group may be more aggressive than in elderly patients.

In the current study, no effect of ART on DSS was seen, which is in concordance with previous studies[10,11].The reason for this may be that in case of tumour recurrence after primary radiotherapy, curatively intended salvage surgery by total laryngectomy is still possible in many cases. In our study, seventy patients (39%) developed local recurrences, of which 58 (83%) were salvaged by a total laryngectomy. Of these 58 patients who were salvaged, only 7 (12%) developed a second local recurrence.

Up to now only a few studies have been published comparing different RT regimes in laryngeal carcinoma only. In a phase III study, Hliniak et al. found an increase in locoregional control of 3-5% in T1-T3 laryngeal carcinoma comparing ART to CF, which however was not statistically significant[12]. In a retrospective study, Garden et al. showed better LC in T2N0 glottic SCC comparing twice daily fractionation with single daily fractionation[13]. Yamazaki et al. reported significantly better LC in T1N0 glottic carcinoma treated with 2.25 Gy per fraction compared to 2 Gy per fraction in a prospective randomised study[14]. Since these studies had different inclusion criteria regarding T-status, N-status and tumour location than our study it is difficult to compare the results.

One of the drawbacks of the current study is that we retrospectively studied the difference between patients treated with ART en CF. As a consequence of this non-randomized historical comparison, significant differences were found with regard to T-status, N-status and primary tumour site. In the past, patients with advanced stage laryngeal carcinoma (T3-T4, N+, mainly supraglottic) were generally treated with surgery with or without postoperative radiotherapy based on the pathologic findings of the resected specimen instead of primary CF radiotherapy. Nowadays, also the more advanced stages are primarily treated with ART when possible, which explains the higher rate of T3-T4, N+ and supraglottic tumours in the ART group compared to the CF group. This negative selection in the ART group might

also explain the higher incidence of tumour related deaths in the ART group, although not statistically significant. This was the main reason to exclude patients with T1, and T2a glottic carcinomas, as these patients were mainly treated with CF in our institution.

CONCLUSION

The results of this retrospective study confirmed that local control in T2-T4 laryngeal carcinoma improved after changing the institutional policy from CF to ART based on the results of a number of prospective randomized studies.

REFERENCES

1. Bourhis J, Overgaard J, Audry H, et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: A meta-analysis. *Lancet*. 2006;368(9538):843-854.
2. Forastiere AA, Pajak TF, Maor M, Weber R. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer (vol 349, pg 2091, 2003). *N Engl J Med*. 2004;350(10):1052-1053.
3. Mendenhall WM, Amdur RJ, Morris CG, Hinerman RW. T1-T2N0 squamous cell carcinoma of the glottic larynx treated with radiation therapy. *J Clin Oncol*. 2001;19(0732-183; 20): 4029-4036.
4. Schrijvers ML, van Riel EL, Langendijk JA, et al. Higher laryngeal preservation rate after CO2 laser surgery compared with radiotherapy in T1a glottic laryngeal carcinoma. *Head Neck*. 2009;31(1097-0347; 1043-3074; 6):759-764.
5. Hartl DM, Ferlito A, Brasnu DF, et al. Evidence-based review of treatment options for patients with glottic cancer. *Head Neck*. 2011(1097-0347; 1043-3074).
6. Overgaard J, Hansen HS, Specht L, et al. Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6 and 7 randomised controlled trial. *Lancet*. 2003;362(1474-547; 9388):933-940.
7. Fu KK, Pajak TF, Trotti A, et al. A radiation therapy oncology group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: First report of RTOG 9003. *Int J Radiat Oncol Biol Phys*. 2000;48(0360-3016; 1):7-16.
8. Egelmeer AG, Velazquez ER, de Jong JM, et al. Development and validation of a nomogram for prediction of survival and local control in laryngeal carcinoma patients treated with radiotherapy alone: A cohort study based on 994 patients. *Radiother Oncol*. 2011;100(1):108-115.
9. Zhang H, Travis LB, Chen R, et al. Impact of radiotherapy on laryngeal cancer survival: A population-based study of 13,808 US patients. *Cancer*. 2011.
10. Horiot JC, Bontemps P, van den Bogaert W, et al. Accelerated fractionation (AF) compared to conventional fractionation (CF) improves loco-regional control in the radiotherapy of advanced head and neck cancers: Results of the EORTC 22851 randomized trial. *Radiother Oncol*. 1997;44(0167-8140; 2):111-121.

11. Bourhis J, Lapeyre M, Tortochaux J, et al. Phase III randomized trial of very accelerated radiation therapy compared with conventional radiation therapy in squamous cell head and neck cancer: A GORTEC trial. *J Clin Oncol*. 2006;24(1527-7755; 0732-183; 18):2873-2878.

12. Hliniak A, Gwiazdowska B, Szutkowski Z, et al. A multicentre randomized/controlled trial of a conventional versus modestly accelerated radiotherapy in the laryngeal cancer: Influence of a 1 week shortening overall time. *Radiother Oncol*. 2002;62(0167-8140; 1):1-10.

13. Garden AS, Forster K, Wong PF, Morrison WH, Schechter NR, Ang KK. Results of radiotherapy for T2N0 glottic carcinoma: Does the "2" stand for twice-daily treatment? *Int J Radiat Oncol Biol Phys*. 2003;55(0360-3016; 0360-3016; 2):322-328.

14. Yamazaki H, Nishiyama K, Tanaka E, Koizumi M, Chatani M. Radiotherapy for early glottic carcinoma (T1N0M0): Results of prospective randomized study of radiation fraction size and overall treatment time. *Int J Radiat Oncol Biol Phys*. 2006;64(0360-3016; 0360-3016; 1):77-82.

Chapter 4

FADD expression as a prognosticator in early stage glottic squamous cell carcinoma of the larynx treated primarily with radiotherapy

Int J Radiat Oncol Biol Phys. 2012;83(4):1220-6

Michiel Schrijvers
Wouter Pattje
Lorian Slagter-Menkema
Mirjam Mastik
Johan Gibcus
Johannes Langendijk
Jacqueline van der Wal
Bernard van der Laan
Ed Schuuring

ABSTRACT

PURPOSE We recently reported on the identification of the Fas-associated death domain (FADD) as a possible driver of the chromosome 11q13 amplicon and the association between increased FADD expression and disease-specific survival in advanced stage laryngeal carcinoma. The aim of this study was to examine whether expression of FADD and its Ser194-phosphorylated isoform (pFADD) predict local control in patients with early stage glottic carcinoma primarily treated with radiotherapy only.

METHODS AND MATERIALS Immunohistochemical staining for FADD and pFADD was performed on pretreatment biopsies of ninety-two patients with T1-T2 glottic squamous cell carcinoma primarily treated with radiotherapy between 1996 and 2005. Cox regression analysis was used to correlate expression levels with local control.

RESULTS High levels of pFADD were associated with a significant better local control (HR 2.40, 95% CI 1.04-5.55, p=0.040). FADD overexpression showed a trend towards better local control (HR 3.656; 95% CI 0.853-15.663, p=0.081). Multivariate Cox regression analysis revealed that high pFADD was the best predictor for local control after radiotherapy.

CONCLUSION This study revealed that expression of phosphorylated FADD is a new prognostic biomarker for a better local control after radiotherapy in patients with early stage glottic carcinomas.

INTRODUCTION

In squamous cell carcinoma of the head and neck region (HNSCC), DNA amplification of the chromosome 11q13 region is observed in approximately 30% of all HNSCC and is therefore one of the most frequently observed genomic abnormalities[1]. In HNSCC, 11q13 amplification is associated with the presence of lymph node metastases, decreased disease-specific survival and decreased overall survival[2]. Recently, we reported that the commonly amplified 11q13 region in oropharyngeal/laryngeal carcinomas contains at least 6 genes, FADD (MORT1), PPFIA1 (LIPRIN), ORAOV1 (TAOS1), FGF19, cortactin (CTTN), cyclin D1 (CCND1) that are amplified and overexpressed in almost all carcinomas with 11q13 amplification[3]. Of all genes in this 11q13 amplicon, FADD was not only amplified the most, but overexpression and amplification of FADD also correlated with increased FADD protein expression suggesting that FADD is a key gene in the 11q13 amplicon[3].

Originally, FADD (Fas (TNFRSF6)-associated via death domain) was reported as a pro-apoptotic adaptor molecule that recruits caspases 8 and 10 to promote formation of the death-inducing signal complex (DISC)[4]. The recruitment of these caspases to the DISC leads to intracellular processing and activation of caspases, eventually resulting in cleavage of downstream targets and apoptosis. More recently, an alternative function for FADD was described as many studies demonstrated that FADD also plays an important role in growth and regulation of the cell cycle[5,6]. Nuclear localization of FADD has been ascribed to FADD phosphorylation at ser194 (referred to as pFADD) and the highest levels of pFADD are observed at the G2/M phase of the cell cycle[7]. In addition, treatment of cells in vitro with agents blocking the G2/M transition resulted in a significant accumulation of pFADD[8,9]. Finally, expression of a ser194-phospho-mimicking FADD mutant caused G2/M cell cycle arrest[9]. All together these data suggest a key role for FADD/pFADD in cell cycle control. Since previous studies showed that cells arrested in the G2/M phase are most radiosensitive[10], FADD is amplified in

more than 30% of HNSCC and pFADD is mainly expressed at the G2/M phase of the cell cycle, we hypothesize that glottic carcinomas with overexpression of pFADD will have better local control after radiotherapy alone.

In a series of 167 advanced-stage oropharyngeal/laryngeal carcinomas, we found that increased levels of both FADD and pFADD were significantly associated with a worse disease-specific survival and positive lymph node status[3,11]. In agreement with this finding in HNSCC, FADD and pFADD overexpression in adenocarcinomas of the lung were both associated with decreased overall survival[12]. In prostate cancer, pFADD expression was associated with progression of disease[9]. In our series 167 of advanced-stage oropharyngeal/ laryngeal carcinomas[3,11], we could not evaluate the prognostic value for local control after radiotherapy because of the presence of many confounding parameters that might influence the clinical outcome such as the variety of different (combined) treatment modalities, different subsites in the head and neck region, and differences in extended disease (e.g. N+) and tumour size. In this paper we examined the prognostic value of FADD and pFADD expression in patients with laryngeal carcinoma primarily treated with radiotherapy alone. To evaluate the effect of FADD/pFADD expression on clinical outcome after radiotherapy we limited the amount of possible interfering variables by selecting a homogeneous study population. Of all patients with laryngeal carcinoma treated at our institution between 1997 and 2004, we selected 92 patients with early-stage (pT1/pT2) glottic carcinoma of the larynx and treated primarily with radiotherapy with curative intent. Since early-stage glottic carcinomas rarely have regional lymph node metastasis on initial diagnosis

and because FADD expression has been associated with the presence of lymph node metastasis in advanced-stage HNSCC as well[11], the analysis of early-stage glottic cancer will restrict our validation to local control after radiotherapy. Immunohistochemical staining of FADD and pFADD on pre-treatment biopsies will be correlated with local control and overall survival.

METHODS AND MATERIALS

Patients and tissues

The selection of patients and samples was described in detail previously[13]. In short, between 1997 and 2004, 638 patients were diagnosed with laryngeal squamous cell carcinoma in the northern part of the Netherlands (comprising >10 medical centres) and treated at our institute. Demographic and clinicopathological data as gender, age, pre-treatment haemoglobin level, T-status, N-status, current and past tobacco use and alcohol use were retrospectively collected by reviewing the patient charts. The inclusion criteria for this study were (1) histologically proven squamous cell carcinoma; (2) localized in the glottis; (3) cT1 and cT2; (4) no evidence for distant metastasis (cM0); (5) curatively treated with radiotherapy alone, and (6) no other previous treatment. Of the 360 patients with T1/T2 glottic carcinomas, 157 formalin-fixed, paraffin embedded pre-treatment biopsies were collected and revised by an experienced pathologist. Tissue specimens with sufficient tumour cells for immuno-histochemical staining were available from 92 patients. The pre-treatment characteristics are

Table 1. Patient characteristics: glottic cancer treated with primarily radiotherapy

	Glottis No. (%) N=92
Sex	
Male	82 (89%)
Female	10 (8%)
Age (years)	
Median (Range)	65 (40-86)
Primary symptom	
Hoarse voice	88 (96%)
Other	4 (4%)
T-status	
1	50 (54%)
2	42 (46%)
N-status	
0	90 (98%)
1	1 (1%)
X	1 (1%)
HB level (mmol/l)	
Median	9.1
Tobacco use past (cigarettes per day)	
0	4 (4%)
1-20	45 (49%)
>20	20 (22%)
unknown	23 (25%)
Tobacco use present (cigarettes per day)	
0	40 (44%)
1-20	29 (32%)
>20	11 (12%)
unknown	12 (13%)
Alcohol use past (U/d)	
0	25 (27%)
1-6	47 (51%)
>6	3 (3%)
unknown	17 (18%)

summarized in Table 1. Informed consent was given by all patients included in the study.

Radiotherapy and follow-up

In all patients radiotherapy was delivered with megavoltage equipment using 6 MV photons as reported previously[13]. T1 tumours were treated with a total dose of 66 Gy using 2 Gy per fraction, 5 times per week. T2 tumours were generally treated with 6 fractions per week to a total dose of 70 Gy in 6 weeks. In case of elective irradiation of the neck nodes, a total dose of 46 Gy was given on the primary PTV with an additional boost of 70 Gy on the primary tumour and pathological lymph nodes.

After completing radiation, patients were followed every 3 months the first and second year, and every 6 months the third, fourth and fifth year. After five years without evidence of disease, patients were discharged from follow-up. Twenty-one patients who developed a local recurrence after radiotherapy were salvaged by total laryngectomy, one patient received palliative therapy because of inoperable recurrent tumour. Nine patients (10%) developed a second primary in the lung (n=4), the head and neck region (n=4) or the colon (n=1). Twenty-six patients

Table 2. Patient characteristics: follow-up

Characteristics	Glottis No. (%) N=92
Events in follow-up	
Any	43 (47%)
Local recurrence	22 (24%)
Regional rec.	3 (3%)
2nd primary	9 (10%)
Death	26 (28%)
Death of disease	7 (27%)
Death not of disease	19 (73%)
Time to first event (months)	
Mean	19
Median (Range)	13 (0-98)
Time to follow-up (months)	
Mean	49
Median (Range)	40 (1-119)
Time to local recurrence (months)	
Mean	14
Median (Range)	11.5 (2-46)
Time to regional recurrence (months)	
Mean	11
Median (Range)	12 (6-16)
Time to 2nd primary (months)	
Mean	26
Median (Range)	10 (0-68)
Time to death (months)	
Mean	33
Median (Range)	28 (3-98)

(28%) died during follow-up, of which 7 by died of disease. All follow-up data are summarized in Table 2.

Immunohistochemistry

Immunohistochemical staining and scoring for FADD, pFADD, cortactin and cyclin D1 was performed as reported previously[3,11] using the murine monoclonal antibody A66-2 (BD Biosciences, Franklin Lakes, NJ), the rabbit polyclonal antibody against Ser^{194} pFADD (BD Biosciences), murine monoclonal antibody 30/ cortactin (BD Biosciences) and the rabbit anti-human monoclonal antibody SP4 (Lab Vision/Neomarkers, Fremont, CA), respectively (see examples in Figure 1). For the scoring of the immunostaining of pFADD and cyclin D1 the percentage of tumour cells with nuclear staining was determined. Cut-off values of percentages for dichotomization for pFADD were determined by using Receiver Operating Curve (ROC) analyses with the best sensitivity/specificity ratio associated with local control was found at a cutoff level of 71%. Percentages of positive staining above the cut-off level were considered as high expression, and those below as negative/low expression. Scoring was performed by two independent observers without knowledge of clinical data. The discordant cases were reviewed by all observers and scores were reassigned on consensus of opinion.

Statistical Analysis

Associations between expression of FADD, pFADD, cyclin D1 and cortactin were analysed using a chi-square test. Follow-up time was calculated from the day of diagnosis until the date of the last follow-up visit. Local recurrence was defined as tumour recurrence at the primary tumour site and was calculated from the date of diagnosis until the day of local recurrence diagnosis or to the last follow-up. Overall survival was defined from the day of diagnosis to the date of death or to last follow-up.

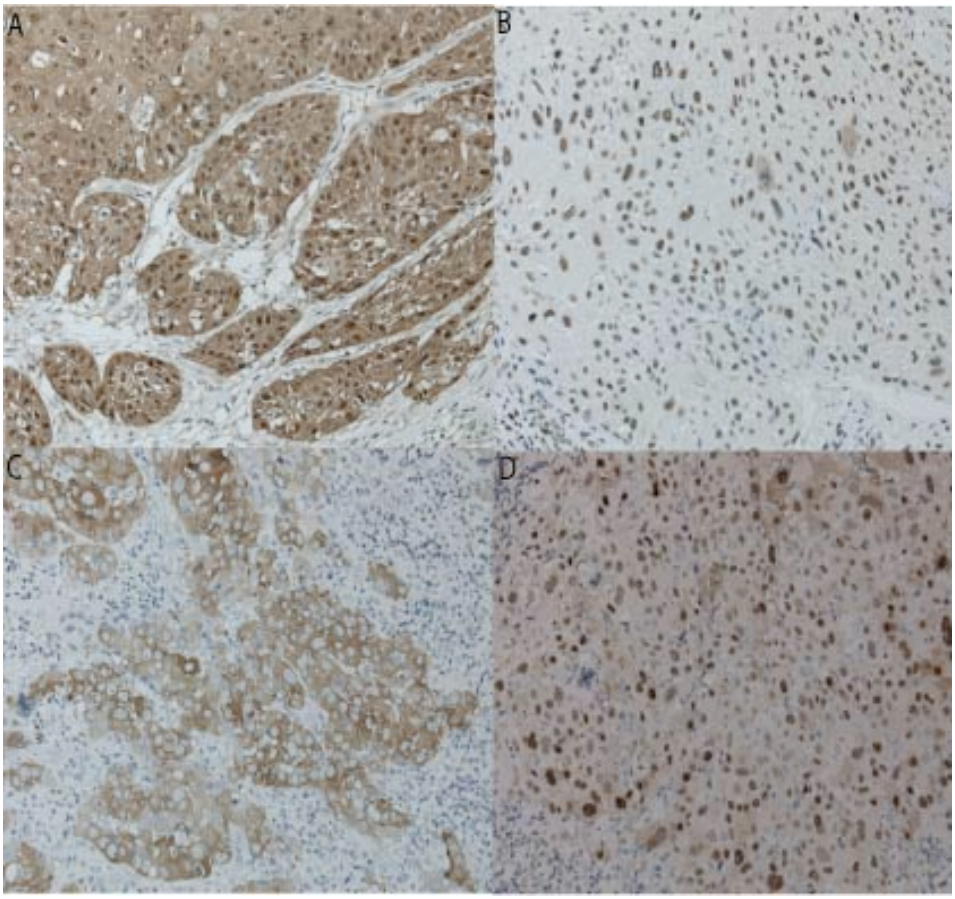

Figure 1. Immunohistochemical staining for FADD, pFADD, cortactin and cyclin D1, magnification 200x. Examples of positive cytoplasmic FADD (A) and cortactin (C), and nuclear pFADD (B) and cyclin D1 (D) staining.

Kaplan-Meier survival analysis and Cox regression analysis adjusted for expression of FADD and pFADD, as well for gender, age, haemoglobin level, T status, N status, tobacco use and alcohol use were performed with local control (LC) and overall survival (OS) as endpoints. Only variables showing an association

with LC or OS in univariate analysis ($p<0.10$) were included in the multivariate Cox regression analysis. Alcohol consumption was excluded from the multivariate analysis because of the large number of missing values (17 missing, 18%). All statistical analysis was performed using the Statistical Package for Social Sciences, version 16.0 (SPSS, Chicago, IL).

RESULTS

FADD and pFADD are not associated with clinicopathological features.

Immunohistochemical staining of FADD was mainly cytoplasmic and very homogeneous across the tumour (Figure 1A). High levels of FADD were detected in 21/92 cases (23%). The immunostaining of pFADD was distributed more heterogeneously within the tumour tissues and predominantly found within the nucleus (Figure 1B). High nuclear pFADD levels were observed in 62/92 cases (67%). No significant association was found between FADD and pFADD expression (results not shown) which is in agreement with the different morphological distributions. No significant associations were found between FADD or pFADD expression and gender, age, haemoglobin level, T stage, N stage, current and past tobacco use and use of alcohol (data not shown).

Expression of FADD and pFADD predicts increased local control in early stage glottic carcinoma.

Univariate Cox regression analysis on dichotomized groups showed that high pFADD expression was associated with better local control (hazard ratio (HR) 2.40, 95% CI 1.04-5.55, $p=0.040$) while borderline significance for the association with this endpoint was found for high FADD expression (HR 3,66; 95% confidence interval (CI) 0.85-15.66, $p=0.081$, Table 3). Kaplan Meier survival analysis showed similar results for both pFADD ($p=0.033$) and FADD ($p=0.060$) (Figure 2A and 2B).

Table 3. Patient and disease characteristics total and related to local recurrence after radiotherapy (N (%); univariate HR, 95% CI, p-value)

Characteristics	Total	Local recurrence	Univariate HR (95% CI)	P-value
FADD expression				
Low	71	20 (28%)	3.656 (0.853-15.663)	0.081
High	21	2 (10%)	1	
pFADD expression				
Low	30	11 (37%)	2.403 (1.041-5.548)	**0.040**
High	62	11 (18%)	1	
Cyclin D1 expression				
Low	43	12 (28%)	1.292 (0.558-2.991)	0.550
High	44	10 (23%)	1	
Cortactin expression				
Low	42	14 (33%)	1.890 (0.762-4.687)	0.169
High	39	7 (18%)	1	
Sex				
Female	10	1 (10%)	1	
Male	82	21 (26%)	2.751 (0.370-20.470)	0.323
Age				
Under age 65	46	11 (24%)	1	
65 and over	46	11 (24%)	1.183 (0.511-2.738)	0.695
Hemoglobine				
High	70	15 (21%)	1	
Low	22	7 (32%)	1.731 (0.704-4.257)	0.232
T-status				
T1	50	9 (18%)	1	
T2	42	13 (31%)	1.906 (0.814-4.463)	0.137
N-status				
N1	1	0 (0%)	1	
N0	91	22 (24%)	20.570 (0.00-1.25 10 8	0.704
Current tobacco use				
Yes	40	8 (20%)	1	
No	40	12 (30%)	1.718 (0.701-4.215)	0.237
Past tobacco use				
No	4	0 (0%)	1	
Yes	65	16 (25%)	21.941 (0.002-295955)	0.524
Alcohol use				
No	25	9 (18%)	1	
Yes	50	13 (31%)	4.816 (1.10-21.087)	**0.037**

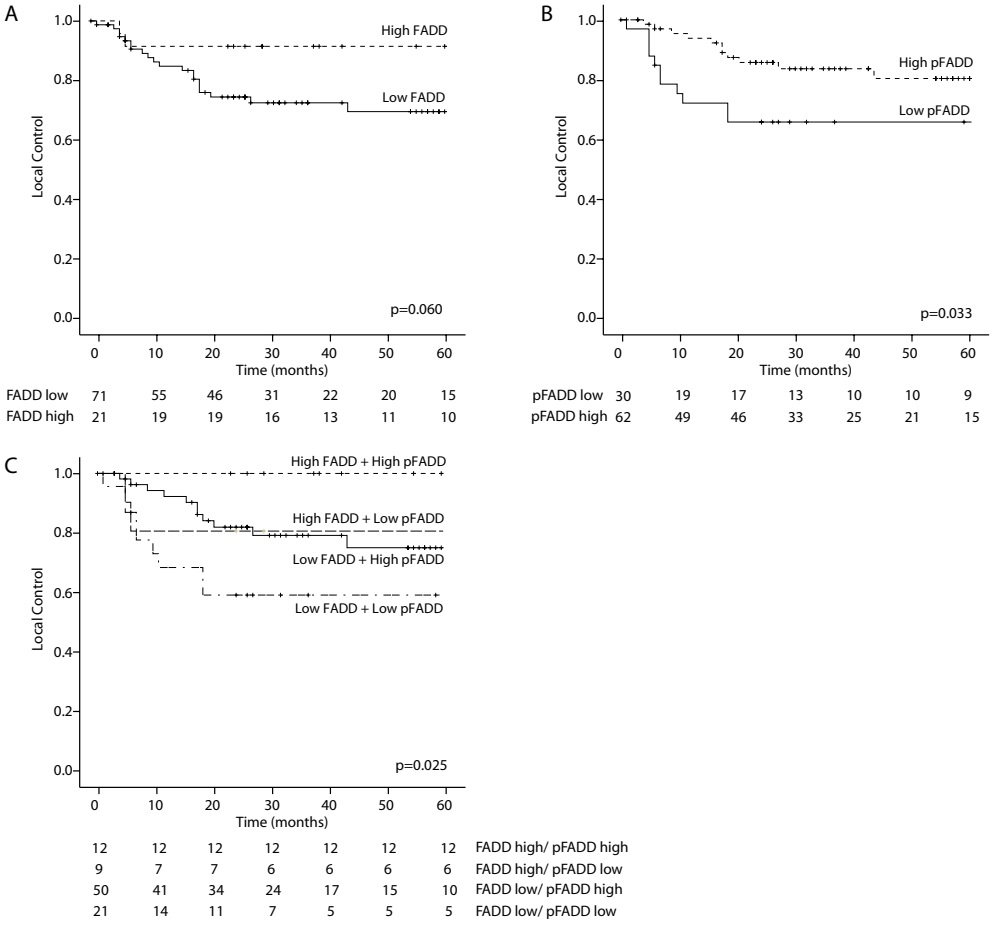

Figure 2. Local control (LC) rate as a function of (A) FADD, (B) pFADD and (C) combination of FADD/pFADD. Patients overexpressing FADD show a trend towards better LC. Patients overexpressing pFADD show a significant better LC. Patients overexpressing both FADD and pFADD have a local control rate of 100%.

Interestingly, none of the 12 patients with high expression of both FADD and pFADD developed a local recurrence, whereas 22 of the 80 patients with low expression of either FADD, pFADD or both (27.5%) developed a local recurrence (p=0.025, Figure 2C). Multivariate Cox regression analysis revealed that high

pFADD was the strongest independent prognostic factor for local control after radiotherapy (HR 2.72, 95% CI 1.17-6.29, p=0.020, table 4).

Table 4. Patient characteristics related to local recurrence after radiotherapy (multivariate HR, 95% CI, p-value)

Characteristics	HR (95% CI)	P-Value
FADD expression		
Low	4.227 (0.981-18.320)	0.053
pFADD expression		
Low	2.715 (1.172-6.287)	**0.020**

Only variables showing a correlation with LC in univariate analysis (p<0.10) were included (pFADD and FADD). Alcohol use was excluded because of the large number of missing values.

Expression of FADD or pFADD does not predict better overall survival

Kaplan Meier survival analysis revealed that high expression of both FADD (p=0.213) and pFADD (p=0.788) were not associated with overall survival (Figure 3A and 3B). Cox regression analysis also revealed that high expression of FADD and pFADD were not associated with overall survival (resp. HR 1.94, 95% CI 0.67-5.65, p=0.223 and HR 1.12, 95% CI 0.49-2.55, p=0.788).

Cyclin D1 and cortactin are not associated with local control

Because cortactin and cyclin D1 are frequently co-amplified and consequently co-overexpressed with FADD in HNSCC[3,11] and have been associated with poor prognosis and/or response to therapy[3,14,15], we immunostained the same series for cortactin and cyclin D1 (see examples in Figure 1C and 1D). Increased expression of cyclin D1 and cortactin was observed in respectively 43 and 39 of 92 cases. Univariate Cox regression analysis (Table 3) and Kaplan Meier survival analysis (Figure 4) showed that high expression of cyclin D1 and cortactin were not associated with local control in early stage glottic cancer.

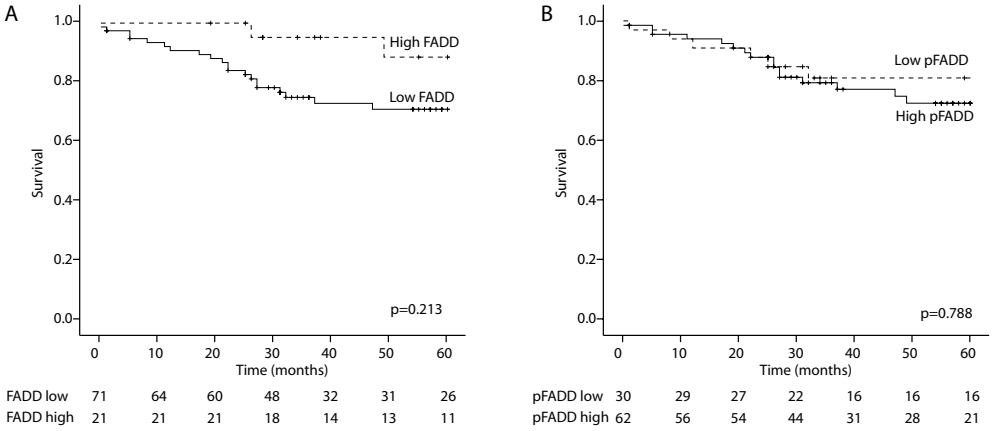

Figure 3. Overall survival (OS) as a function of (A) FADD and (B) pFADD. No significant correlation was found between either FADD or pFADD overexpression and OS.

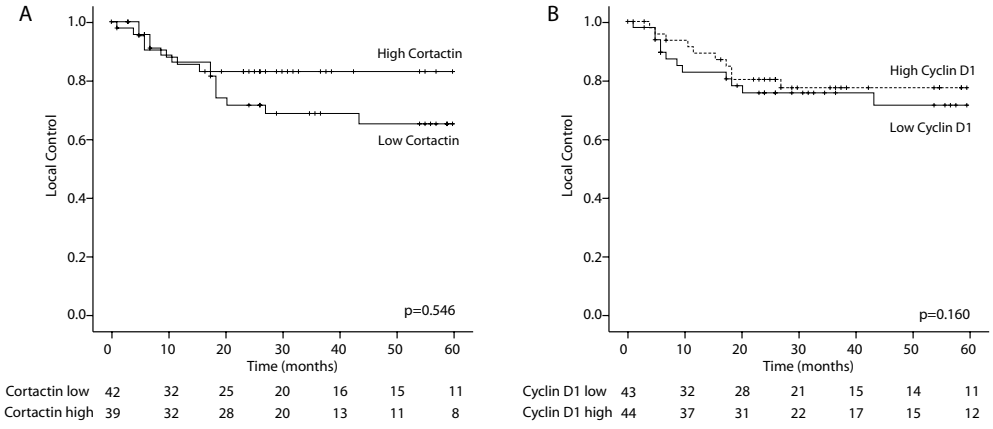

Figure 4. Local control (LC) as a function of (A) cortactin and (B) cyclin D1. No significant correlation was found.

DISCUSSION

Laryngeal squamous cell carcinoma is the most common type of HNSCC and accounts for approximately 20% of all newly diagnosed HNSCC. Nowadays most patients with T1/T2 stage laryngeal carcinoma are treated with radiotherapy because of better laryngeal function after treatment compared to surgery. The 5-year LC and OS rates for T1/T2 laryngeal carcinoma vary between 69%-94% (LC) and 63%-82% (OS)[16]. Unfortunately, except for TNM status, no good clinico-pathological factors are available to predict clinical outcome in early stage laryngeal carcinoma treated with radiotherapy. The clinical relevance is obvious, because in case of recurrent disease after radiotherapy, salvage laryngectomy is mandatory resulting in loss of vocal cord function and therefore quality of live. Therefore, molecular tumour markers could be useful to predict local recurrence and survival in early stage glottic carcinoma treated with radiotherapy.

Associations between expression of either FADD or pFADD with clinical outcome have been described in different tumour types. Chen and coworkers showed that FADD and pFADD over-expression were both associated with a decreased OS in patients with adenocarcinoma of the lung[12]. Shimada and coworkers reported that expression of pFADD was associated with progression of prostate carcinoma[9]. In a previous study we found an association between FADD and pFADD expression and clinical outcome in a group of mainly advanced stage laryngeal carcinomas[3,11]. No associations could be performed between FADD/ pFADD expression and response to therapy because of the heterogeneity of the patient series. In this paper we selected a homogeneous series of patients with T1/ T2 glottic carcinoma treated with radiotherapy only. Our data revealed that high expression of FADD and even more significantly pFADD overexpression was associated with better local control. No association with overall survival was found. These data strongly suggest that overexpression of pFADD is associated with better radiosensitivity.

FADD is part of the chromosome 11q13 amplicon[3]. We have shown that not only FADD but also several other genes in the amplicon (PPFIA1, TPCN2, FLJ442258, ORAOV1, FGF19, CTTN/cortactin, CCND1/cyclin D1) are overexpressed in HNSCC carcinomas with 11q13 amplification[3]. Two of these genes (cyclin D1 and cortactin) have been studied extensively[1]. Cyclin D1 has been associated with response to radiotherapy previously in breast cancer both in a clinical study[15,17,18] and an in vitro model[14],and cortactin with worse clinical outcome in HNSCC[11] and cell migration in vitro[19]. Immunostaining of the same series of 92 glottic cancers revealed no association between either cortactin or cyclin D1 expression and local control (Table 3 and Figure 4). The lack of this association for cyclin D1 is not consistent with the observed response to therapy in breast cancer, but concordant with studies in HNSCC that failed to associate cyclin D1 expression and clinical outcome[20]. In this study we only selected early stage (T1/T2) glottic carcinomas, in which regional or distant metastasis are relatively low (only 1/92) which results from the less developed lymphatic drainage system of the glottic larynx. The lack of the observed association between cortactin and local control in this series of early stage glottic carcinoma is therefore in good agreement with its function in cell migration and invasion[19]. This analysis showed that not cyclin D1 and/or cortactin, but high pFADD expression is associated with local control in our series of early stage glottic carcinomas.

Our data also suggest that the expression of pFADD might mediate the sensitivity of tumour cells to radiotherapy. However, at this moment we can only speculate how. Besides the role of FADD in apoptosis, more recently phosphorylation of FADD (pFADD) at serine 194 was reported to be involved in cell cycle progression[4,5,7,8]. Hua et al. showed that phosphorylation of FADD is essential for growth/proliferation in T cells[5]. Shimada and Alappat et al. have shown that phosphorylation of FADD caused more cells to be arrested in the G2/M phase of the cell cycle[8,9]. A possible explanation for this is that cells in the G2/M phase are more radiosensitive than cells in other phases of the cell cycle[10]. Furthermore, the fact that phosphorylation of FADD is associated with nuclear

localisation in our tumours and involved in G2/M arrest in in-vitro models[9], suggests that FADD plays an important role in sensitizing these tumours for radiation. However, whether pFADD itself triggers cells into G2/M arrest or cells at G2/M arrest show expression of pFADD has to be tested in future experiments by knocking-down or expressing FADD and pFADD mutants in HNSCC cell lines and determine directly RT response by the classical clonogenic survival assay.

CONCLUSION

In summary, we showed that increased expression of pFADD/FADD is associated with a better local control in early stage glottic carcinoma treated with radiotherapy alone. A possible explanation is that pFADD expression is associated with more cells arrested in the radiosensitive G2/M phase of the cell cycle. Our findings suggest that pFADD might be new prognostic biomarkers to predict local recurrence in T1/T2 glottic carcinoma treated with radiotherapy.

REFERENCES

1. Schuuring E. The involvement of the chromosome 11q13 region in human malignancies: Cyclin D1 and EMS1 are two new candidate oncogenes--a review. *Gene*. 1995;159(0378-1119; 1): 83-96.
2. Akervall JA, Jin Y, Wennerberg JP, et al. Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck. *Cancer*. 1995;76(0008-543; 5):853-859.
3. Gibcus JH, Menkema L, Mastik MF, et al. Amplicon mapping and expression profiling identify the fas-associated death domain gene as a new driver in the 11q13.3 amplicon in laryngeal/ pharyngeal cancer. *Clin Cancer Res*. 2007;13(1078-0432; 21):6257-6266.
4. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of fas and initiates apoptosis. *Cell*. 1995;81(4):505-512.
5. Hua ZC, Sohn SJ, Kang C, Cado D, Winoto A. A function of fas-associated death domain protein in cell cycle progression localized to a single amino acid at its C-terminal region. *Immunity*. 2003;18(1074-7613; 4):513-521.
6. Osborn SL, Sohn SJ, Winoto A. Constitutive phosphorylation mutation in fas-associated death domain (FADD) results in early cell cycle defects. *J Biol Chem*. 2007;282(0021-9258; 0021-9258; 31):22786-22792.
7. Alappat EC, Feig C, Boyerinas B, et al. Phosphorylation of FADD at serine 194 by CKIalpha regulates its nonapoptotic activities. *Mol Cell*. 2005;19(1097-2765; 3):321-332.
8. Alappat EC, Volkland J, Peter ME. Cell cycle effects by C-FADD depend on its C-terminal phosphorylation site. *J Biol Chem*. 2003;278(0021-9258; 43):41585-41588.
9. Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Konishi N. Phosphorylation status of fas-associated death domain-containing protein (FADD) is associated with prostate cancer progression. *J Pathol*. 2005;206(0022-3417; 4):423-432.
10. Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys*. 2004;59(0360-3016; 4):928-942.
11. Gibcus JH, Mastik MF, Menkema L, et al. Cortactin expression predicts poor survival in laryngeal carcinoma. *Br J Cancer*. 2008;98(0007-0920; 5):950-955.

12. Chen G, Bhojani MS, Heaford AC, et al. Phosphorylated FADD induces NF-kappaB, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. *Proc Natl Acad Sci U S A*. 2005;102(35):12507-12512.

13. Schrijvers ML, van der Laan BF, de Bock GH, et al. Overexpression of intrinsic hypoxia markers HIF1alpha and CA-IX predict for local recurrence in stage T1-T2 glottic laryngeal carcinoma treated with radiotherapy. *Int J Radiat Oncol Biol Phys*. 2008;72(0360-3016; 0360-3016; 1): 161-169.

14. Coco Martin JM, Balkenende A, Verschoor T, Lallemand F, Michalides R. Cyclin D1 overexpression enhances radiation-induced apoptosis and radiosensitivity in a breast tumor cell line. *Cancer Res*. 1999;59(0008-5472; 0008-5472; 5):1134-1140.

15. Yoo SS, Carter D, Turner BC, et al. Prognostic significance of cyclin D1 protein levels in early-stage larynx cancer treated with primary radiation. *Int J Cancer*. 2000;90(1):22-28.

16. Mendenhall WM, Werning JW, Hinerman RW, Amdur RJ, Villaret DB. Management of T1-T2 glottic carcinomas. *Cancer*. 2004;100(9):1786-1792.

17. Xia F, Powell SN. The molecular basis of radiosensitivity and chemosensitivity in the treatment of breast cancer. *Semin Radiat Oncol*. 2002;12(1053-4296; 1053-4296; 4):296-304.

18. Akervall JA, Michalides RJ, Mineta H, et al. Amplification of cyclin D1 in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin D1 overexpression. *Cancer*. 1997;79(0008-543; 2):380-389.

19. van Rossum AG, Moolenaar WH, Schuuring E. Cortactin affects cell migration by regulating intercellular adhesion and cell spreading. *Exp Cell Res*. 2006;312(0014-4827; 0014-4827; 9): 1658-1670.

20. Morshed K, Skomra D, Korobowicz E, Szymanski M, Polz-Dacewicz M, Golabek W. An immunohistochemical study of cyclin D1 protein expression in laryngeal squamous cell carcinoma. *Acta Otolaryngol*. 2007;127(0001-6489; 7):760-769.

Chapter 5

Overexpression of the intrinsic hypoxia markers HIF1α and CA-IX predict local recurrence in T1/T2 glottic laryngeal carcinoma treated with radiotherapy

Int J Radiat Oncol Biol Phys. 2008;72(1):161-9

Michiel Schrijvers
Bernard van der Laan
Truuske de Bock
Wouter Pattje
Mirjam Mastik
Lorian Menkema
Johannes Langendijk
Philip Kluin
Ed Schuuring
Jacqueline van der Wal

ABSTRACT

PURPOSE To examine the prognostic value of three endogenous hypoxia markers (HIF1α, CA-IX and GLUT-1) on clinical outcome in patients with early stage glottic carcinoma primarily treated with radiotherapy, and to find a predictive hypoxic profile to choose the optimal treatment in early stage laryngeal carcinoma.

METHODS AND MATERIALS Immunohistochemistry for HIF1α, CA-IX and GLUT-1 was performed on formalin-fixed paraffin-embedded pre-treatment tissue samples of 91 glottic squamous cell carcinomas. The patient group consisted only of early stage (T1/T2) glottic carcinomas, and all patients were treated with radiotherapy only. Relative tumour staining was scored on the tissue samples. ROC analysis was performed to determine the optimal cutoff value for each tumour marker. Cox regression analyses for the variables HIF1α, CA-IX, GLUT-1, sex, age, haemoglobin level, T-status, N-status, tobacco use and alcohol use were performed with local control and overall survival as endpoints.

RESULTS HIF1α overexpression in early stage glottic carcinoma significantly correlated with worse local control (HR 3.05, p=0.021) and overall survival (HR 2.92, p=0.016). CA-IX overexpression was significantly correlated with worse local control (HR 2.93, p=0.020). GLUT-1 overexpression did not show any correlation with clinical outcome parameters.
Tumours showing a non-hypoxic profile (defined as low HIF1α and low CA-IX) were significantly correlated to a better local control (HR 6.32, p=0.013).

CONCLUSIONS Early stage glottic laryngeal carcinomas with low expression of both HIF1α and CA-IX are highly curable with radiotherapy, for this group radiotherapy is a good treatment option. For tumours with HIF1α or CA-IX overexpression hypoxic modification before radiotherapy or primary surgical treatment should be considered.

INTRODUCTION

Head and neck cancer is the sixth most prevalent cancer in the world with an incidence of 700.000 cases per year[1]. Twenty percent of all head and neck tumours originate in the larynx, of which the majority is located in the glottic region. Most patients with glottic carcinoma present with early stage disease (T1/T2).

Optimal management of early stage glottic carcinomas requires both optimal tumour treatment as well as maximal preservation of laryngeal function such as swallowing and quality of the voice. Radiotherapy is a generally accepted treatment modality which meets both of these criteria. Besides primary radiotherapy, nowadays more advanced surgical techniques have been developed, such as partial laryngectomy or endoscopic laser surgery. Comparing surgery and radiotherapy as primary treatment revealed no differences in local control rates and overall survival in patients with early stage glottic carcinoma [2,3]. However, presently, no randomised controlled trials have been performed comparing surgery and radiotherapy as primary treatment in early stage glottic laryngeal carcinoma. Surgery will lead to less optimal laryngeal function, especially in the more extensive T1 and T2 tumours, due to excision of delicate voice producing elements in the larynx. Therefore, in these patients, outcome regarding speech and voice quality are significantly better when treated with primary radiotherapy[2]. Because of the good preservation of laryngeal function, in many countries, including the Netherlands, primary radiotherapy has become the standard therapy for early stage glottic carcinomas. In the Netherlands in 2003, 675 laryngeal carcinomas were diagnosed (www.ikcnet.nl). In the Netherlands, the local control rate for T1/T2 laryngeal carcinoma obtained with primary radiotherapy varies between 75% and 95%, comparable to the local control rates obtained with primary surgery alone[3]. In case of a local recurrence, partial laryngectomy in a previously irradiated area is not considered in most cases, because of the high probability of postoperative complications, such as wound healing problems. Therefore the majority of patients will be salvaged by total laryngectomy, which, however, may have a negative

impact on health-related quality of life. Because partial laryngectomy may serve as an alternative primary treatment modality for early glottic carcinoma, prediction of patients that are likely to develop a local recurrence after primary radiotherapy may be useful in order to select the most optimal treatment in individual patients.

At present, there are no well-defined and consistent clinical characteristics, pathological features and/or molecular markers that are able to predict outcome in terms of local control after primary radiotherapy accurately.

Tumour hypoxia is a well-known prognostic factor with regard to local control after primary radiotherapy. In various types of cancer, including head and neck squamous cell carcinoma (HNSCC), hypoxia is associated with worse loco-regional control and survival after radiotherapy as a result of decreased radiosensitivity[4-6]. Several techniques are available to measure tumour hypoxia, including clinical invasive measurement using needle electrodes, and less invasive methods like exogenous markers (pimonidazole and EF5) and endogenous hypoxia markers (HIF1α, HIF2a, GLUT-1, GLUT-3, CA-IX)[7,8]. Of all intrinsic hypoxia markers, *Hypoxia Inducible Factor 1a* (HIF1α), *Carbonic Anhydrase IX* (CA-IX) and *Glucose Transporter 1* (GLUT-1) have been studied the most in a variety of tumours, including tumours of the breast, cervix, lung and head and neck and overexpression is associated with worse clinical outcome[9-11]. Some investigators showed that upregulation of HIF1α, a transcriptional factor, leads to upregulation of CA-IX and GLUT-1. Therefore we hypothesized that the combined assessment of HIF1α, CA-IX and GLUT-1 would provide a more powerful prediction of local recurrence than the assessment of a single factor. So far, only a few studies investigated the predictive value of various combinations of intrinsic hypoxia markers with regard to clinical outcome. Hui et al. have reported a positive correlation between HIF1α and CA-IX in a group of patients with nasopharyngeal carcinoma**,** with worse progression-free survival when both markers are overexpressed[12]. However, none of these studies reported on the combined assessment of HIF1α, CA-IX and GLUT-1[12,13].

Therefore, the aim of this study was to identify those carcinomas which have a high probability on local recurrence after radiotherapy by examining the prognostic value of a combination of the intrinsic hypoxic markers HIF1α, CA-IX and GLUT-1 on local recurrence. Besides these hypoxic markers, we also examined classical prognostic markers of clinical outcome such as age, gender, TNM stage, pre-treatment haemoglobin level, alcohol and smoking history[14]. For this purpose we have constructed a homogenous subgroup of HNSCC, consisting only of early stage (T1/T2) carcinomas of the glottic larynx, all treated primarily with radiotherapy. Immunohistochemical staining for HIF1α, CA-IX and GLUT-1 was performed and scoring intensity was correlated with clinical outcome parameters.

METHODS AND MATERIALS

Patients and tissues

Between 1997 and 2004, 638 patients were diagnosed with laryngeal squamous cell carcinoma in the northern part of the Netherlands (comprising >10 medical centers) at the University Medical Center Groningen (UMCG) including 433 glottic, 186 supraglottic, 8 subglottic and 11 transglottic tumours. Of the 433 glottic, 360 (83%) were T1/T2. Demographic and clinicopathological data as gender, age, pre-treatment haemoglobin level, T-status, N-status, current and past tobacco use and alcohol use were retrospectively collected by reviewing the patient charts. The inclusion criteria for this study were histologically proven squamous cell carcinoma of the glottic larynx, staged as T1/T2 and curatively treated with radiotherapy. In this study we included the pre-treatment paraffin embedded tumour material from 3 large medical centers in the northern part of the Netherlands: the University Medical Center Groningen, the Wilhelmina Hospital Assen and the Scheper Hospital Emmen (n = 157 patients). Of these 157 patients, paraffin embedded pre-treatment biopsy material with sufficient carcinoma cells for immunohistochemical staining was available of 91 patients consisting of 47 T1

(52%) and 44 T2 (48%). The median age of the patients was 66 years (range 40-84). The pre-treatment characteristics are presented in Table 1. After completing the treatment, patients were followed every 3 months the first and second year, and every 6 months the third, fourth and fifth year. After five years of disease free follow-up, patients were discharged from follow-up. Most patients who developed a local recurrence after radiotherapy were salvaged by total laryngectomy.

Formalin-fixed, paraffin-embedded, pre-treatment biopsy material was collected of all 91 patients that fulfilled the inclusion criteria. All pre-treatment biopsy slides were revised and tumour percentage was estimated by an experienced pathologist (JvdW). Informed consent was given by all patients included in the study.

Table 1. Clinical and pathological characteristics

	Glottis No. (%) N=91
Sex	
Male	81 (89%)
Female	10 (11%)
Age (years)	
Median (Range)	66 (40-84)
Primary symptom	
Hoarse voice	87 (96%)
Other	4 (4%)
T-status	
1	47 (52%)
2	44 (48%)
N-status	
0	88 (97%)
1	2 (2%)
X	1 (1%)
M-Status	
0	0 (100%)
HB level (mmol/l)	
Median (Range)	9.1 (7.3-10.6)
HIF1a Level	
High HIF1a	46 (51%)
Low HIF1a	45 (49%)
CA-IX Level	
High CA-IX	39 (43%)
Low CA-IX	52 (57%)
GLUT-1 Level	
High GLUT-1	53 (58%)
Low GLUT-1	38 (42%)

Continued on next page

Radiotherapy

Radiotherapy was delivered using megavoltage equipment (6 MV linear accelerator). In T1 and T2 with normal vocal cord mobility, the target volume encompassed the gross tumour volume (GTV) with margins of at least 1 cm for the clinical target volume (CTV) of the primary field. In case of T2N0 with impaired vocal cord mobility, the CTV also encompassed elective nodal levels (II-IV) at both sides. In

case of N+, the CTV was extended to level Ib-V of the involved neck. The CTV of the boost encompassed the GTV with at least a 0.5 cm margin. The planning target volume (PTV) included the CTV plus a 0.5 cm margin. Generally, these target volumes were irradiated using two opposing lateral fields. In case of primary radiotherapy for T1 tumours, the total dose was 66 Gy, using 2 Gy per fraction, 5 times per week. T2 tumours were generally treated with 6 fractions per week with a second fraction on Friday afternoon with a minimum interval of 6 hours, to a total dose of 70 Gy in 6 weeks. In case of elective irradiation of the neck nodes, a total dose of 46 Gy was given on the primary PTV with an additional boost of 70 Gy on the primary tumour and pathological lymph nodes.

Table 1. Continued

	Glottis No. (%) N=91
Tobacco use present (per day)	
0	39 (43%)
1-20	29 (32%)
>20	12 (13%)
unknown	11 (12%)
Tobacco use past (per day)	
0	4 (4%)
1-20	45 (50%)
>20	22 (24%)
unknown	20 (22%)
Alcohol use past (per day)	
0	22 (24%)
1-6	50 (55%)
>6	3 (3%)
Unknown	16 (18%)

Immunohistochemistry

The HIF1α and GLUT-1 proteins in the tissue sections were detected using the murine monoclonal antibodies Clone 54 (BD Biosciences, NJ, USA) and the polyclonal rabbit anti-human GLUT-1 antibody (DakoCytomation, Glostrup, Denmark) respectively, according to instructions of the manufacturers. The CA-IX protein was detected using the murine monoclonal antibody M75 (Kind gift by Jaromir Pastorek, University of Bratislava, Slovakia)[15]. Briefly, HIF1α, CA-IX and GLUT-1 staining was performed on 4 μm paraffin sections. Slides were deparaffinized in xylene twice for 10 minutes and subsequently rehydrated through a series of decreasing ethanol dilutions and PBS. Antigen retrieval was achieved by heating in a microwave in pre-heated Tris/EDTA buffer (pH 9.0, HIF1α) or

citratebuffer (pH 6.0, Glut-1) for 15 minutes at a temperature of 100°C. For CA-IX, no antigen retrieval was performed. To block endogenous peroxidase activity, H_2O_2 0.3% was applied for 30 minutes at room temperature. Slides were washed 3 times with PBS afterwards. Slides were stained for 1 hour with antibodies against CA-IX (M75, 1:500), GLUT-1 (rabbit polyclonal anti-human , 1:100), or stained overnight 4 °C with a murine monoclonal antibody against HIF1α (Clone 54, 1:100). Secondary antibodies RAM^{BIO} (Dakocytomation, Glostrup, Denmark) (HIF1α and CA-IX) and GAR^{BIO} (Dakocytomation, Glostrup, Denmark) (GLUT-1) were diluted 1:300 in 1% BSA/PBS complemented with 1% human AB serum and applied for 30 minutes at room temperature. Tertiary antibody ABC^{HRP} 1:100 (Dakocytomation, Glostrup, Denmark) (HIF1α, CA-IX, GLUT-1) was applied for 30 minutes at room temperature. The peroxidase reaction was performed by applying 3, 3' diaminobenzide tetrachlodride (DAB) for 10 minutes and after washing with PBS the slides were counterstained for 2 minutes with haematoxylin and fixated.

Scoring Method

After having set the scoring method with an experienced pathologist, all slides were evaluated by 2 teams separately who were both blinded to clinical outcome. Differences in scores between the two teams were resolved at a conference microscope.

The percentage of positive tumour cells for each antibody was scored. For HIF1α (Fig.1a), only nuclear staining, and for CA-IX (Fig.1b) and GLUT-1 (Fig. 1c) only membranous staining were scored as positive, as reported previously[12,16]. The intensity for all three antibodies was relatively homogeneous, and therefore not incorporated in the scoring method. Cut-off values of percentages for dichotomization of the data were determined for each staining individually by using Receiver Operating Curve (ROC) analyses[17]. The optimum between sensitivity and specificity related to local recurrence was chosen as the strongest deviation from the reference line. Tumours with a percentage of positive staining

above the cut-off level were considered as high expression, and those below as negative/low expression. For HIF1α the best sensitivity/specificity ratio was found at a cut-off percentage of 0.5%, for CA-IX 12.5% and for GLUT-1 35%. We decided to define tumours showing a high expression of HIF1α or CA-IX, or both as hypoxic tumours.

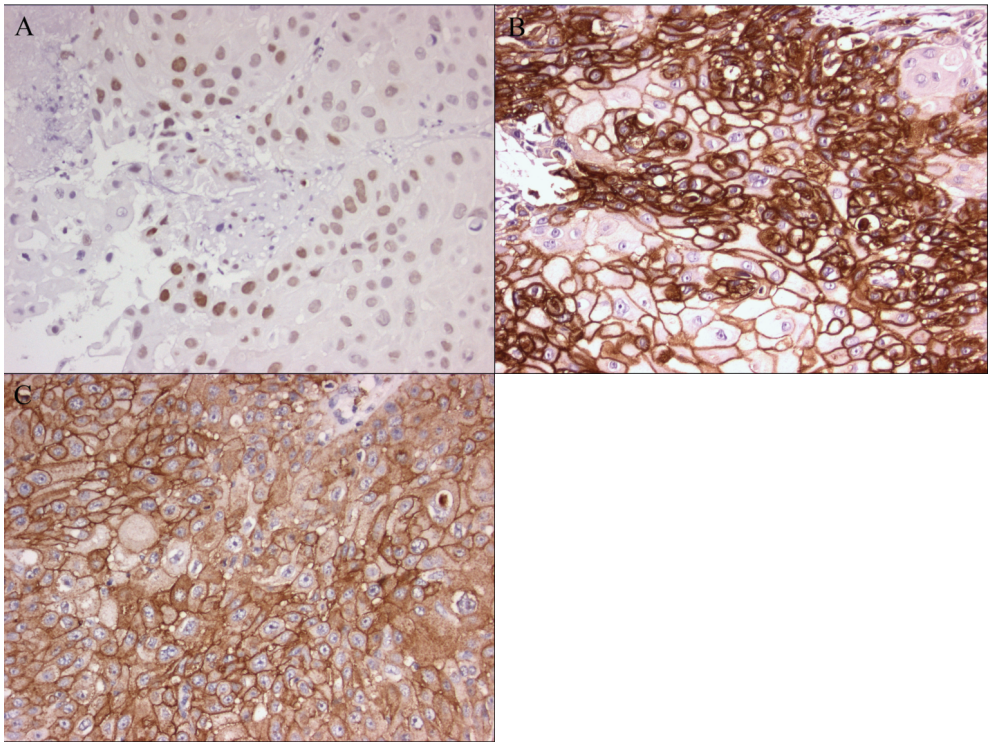

Figure 1. Immunohistochemical staining for HIF1α (nucleus), CA-IX and GLUT-1 (both membranous). Magnification 200x. Nuclear HIF1α (A), membraneus CA-IX (B) and GLUT-1 (C) staining.

Statistical analysis

Associations between HIF1α, CA-IX and GLUT-1 expression and the other pre-treatment parameters (gender, age, haemoglobin level, T-status, N-status, tobacco

use and alcohol use) were analysed using a chi-square test. Follow-up time was calculated from the day of diagnosis until the date of the last follow-up. Local recurrence was defined as tumour recurrence at the primary tumour site, and was calculated from the date of diagnosis until the day of local recurrence or last follow-up. Overall survival was defined as the day of diagnosis until the day of death or last follow-up.

Kaplan-Meier survival analysis and Cox regression analysis for the variables HIF1α, CA-IX, GLUT-1, a combination of the intrinsic hypoxia markers as well as for sex, age, haemoglobin level, T-status, N-status, tobacco use and alcohol use was performed with local control and overall survival as endpoints. Only variables showing a significant relation with clinical outcome in univariate analysis ($p<0.05$) or showing a trend towards significance ($p<0.10$) were included in a multivariate Cox regression model. Because of the limited number of local recurrences (n=21) and deaths (n=26) in our study we included a maximum of 2 variables in the multivariate analysis, according to the study by Peduzzi et al[18]. Alcohol consumption was excluded from multivariate analysis because of the large number of missing values (16 missing, 17.5%).

Because CA-IX and GLUT-1 are two of the genes up-regulated by HIF1α, we also investigated the relationship between HIF1α and CA-IX or GLUT-1 with a Pearson's correlation test. All statistical analysis was performed using the statistical package SPSS 14.0.0 (SPSS, inc., Chicago, IL, USA).

RESULTS

Follow-up data

At the date of analysis, 21 (23%) patients had a local recurrence. Of the 21 patients with a local recurrence, 19 were treated with a total laryngectomy, one patient did not receive any treatment because of tumour involvement of the oesophagus and trachea, and one patient died of disease before treatment was given. Seven (8%) patients developed a second primary tumour. Five of these tumours occurred in the

lung, and two in the head and neck region. Of the 91 patients, 26 patients died (29%). Of these 26 patients, 8 patients died of their index tumour. Follow-up data are presented in Table 2.

Table 2. Follow-up data

Characteristics	Glottis No. (%) N=91
Events in follow-up	
Local recurrence	21 (23%)
Regional rec.	4 (4%)
2nd primary	7 (8%)
Death	26 (29%)
DOD	8 (31%)
DNOD	18 (69%)
Time to first event (months)	
Median (Range)	17 (3-68)
Time to follow-up (months)	
Median (Range)	40 (1-119)
Time to local recurrence (months)	
Median (Range)	12 (5-46)
Time to regional recurrence (months)	
Median (Range)	14 (6-27)
Time to 2nd primary (months)	
Median (Range)	21 (9-68)
Time to death (months)	
Median (Range)	29 (3-98)

Association between pre-treatment variables

No significant associations were found between HIF1α, CA-IX and GLUT-1 expression and the following pre-treatment parameters: gender, age, haemoglobin, T-status, N-status, current tobacco use, past tobacco use and use of alcohol (P>0.05, data not presented). Pearson's correlation test showed a significant 2-sided correlation between HIF1α expression and CA-IX expression (r=0.28, p=0.008) as was found in previous studies[12,19]. No significant correlation between GLUT-1 and HIF1α was found.

Low HIF1α and CA-IX expression predict a high local control rate

Univariate Cox regression analysis showed that high HIF1α (HR 3.05, 95% CI 1.18-7.86) and CA-IX (HR 2.93, 95% CI 1.18-7.26) expression predicted worse local control (Table 3). Kaplan Meier survival curves showed this as well (Fig. 2a + 2b). High GLUT1 (HR 1.94, 95% CI 0.82-4.61) expression did not predict for worse local control (Table 3 and Fig. 2c). Of all other baseline parameters, only high alcohol consumption (HR 8.72, 95% CI 1.16-65.80) predicted a worse local

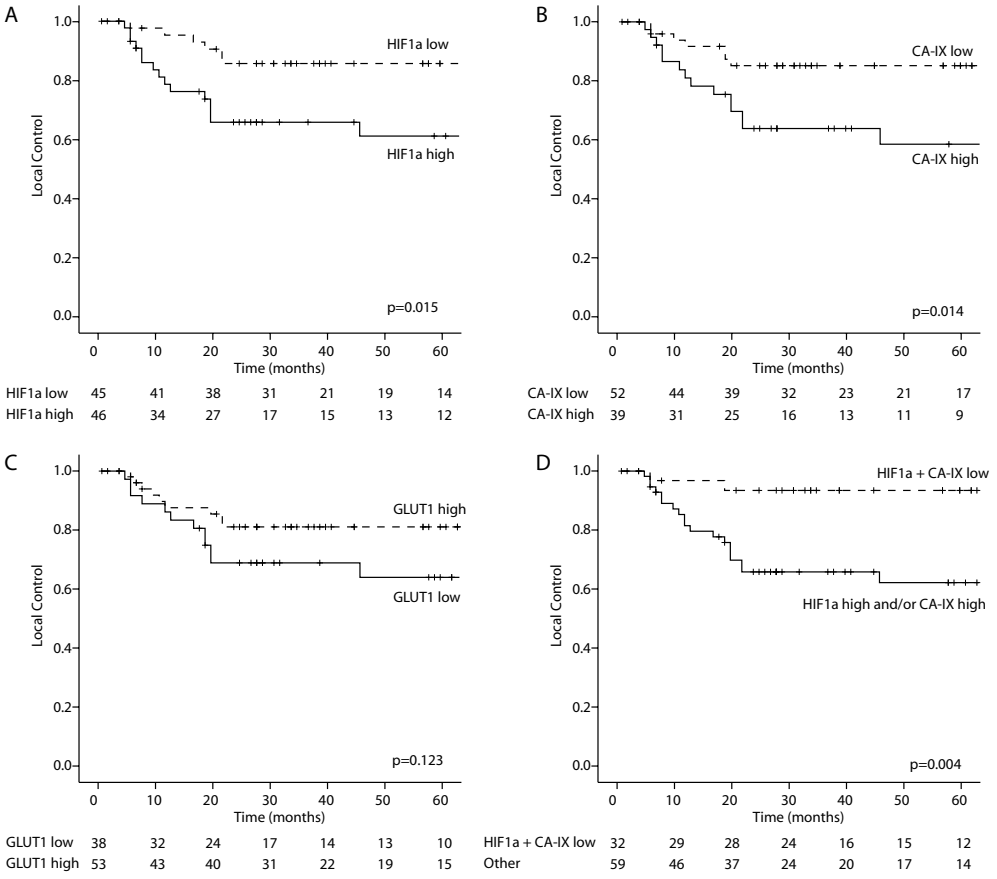

Figure 2. Local control rate as function of (A) HIF1α; (B) CA-IX; (C) GLUT1; (D) HIF1α + CA-IX expression. Tumours overexpressing HIF1α and CA-IX show a significantly worse local control rate (A + B). Tumours overexpressing GLUT1 show a trend towards better local control (C). Tumours showing low expression of both HIF1α and CA-IX show a significantly better local control (D).

control, and a low pre-treatment haemoglobin level (HR 2.23, 95% CI 0.92-5.41) showed a trend towards a worse local control (Table 3).

We decided to define tumours showing a high expression of HIF1α or CA-IX, or both as hypoxic tumours. In our study population, 59 out of 91 (65%) patients showed a hypoxic pattern. Of these 59 patients, 19 of the 59 (32%) patients

Table 3. Patient and disease characteristics total and related to local recurrence after radiotherapy (N (%); univariate Hazard Ratio, 95% CI, p-value)

Characteristics	Total	Local recurrence	Univariate HR (95% CI)	P-value
Level of HIF1α expression				
Low	45	6 (13%)	1	
High	46	15 (33%)	3.05 (1.18-7.86)	**0.021**
Level of CA-IX expression				
Low	52	7 (14%)	1	
High	39	14 (36%)	2.93 (1.18-7.26)	**0.020**
Level of GLUT-1 expression				
High	38	9 (17%)	1	
Low	53	12 (32%)	1.94 (0.82-4.61)	0.132
HIF1α + CA-IX expression				
Both low	32	2 (6%)	1	
Both high or combination	59	19 (32%)	6.32 (1.47-27.15)	**0.013**
Sex				
Female	10	1 (10%)	1	
Male	81	20 (25%)	2.57 (0.34-19.14)	0.358
Age				
Under age 66	48	11 (23%)	1	
66 and over	43	10 (23%)	1.20 (0.51-2.83)	0.678
Hemoglobine				
High	68	13 (19%)	1	
Low	23	8 (35%)	2.23 (0.92-5.41)	0.075
T-status				
T1	47	8 (17%)	1	
T2 or more	44	13 (30%)	1.84 (0.76-4.43)	0.176
N-status				
N1	2	0 (0%)	1	
N0	88	21 (24%)	21.06 (0.0-1862347)	0.60
Current tobacco use				
Yes	41	10 (24%)	1	
No	39	10 (26%)	1.164(0.48-2.80)	0.734
Past tobacco use				
No	4	0 (0%)	1	
Yes	67	17 (25%)	21.89 (0.002-245038)	0.516
Alcohol use				
No	22	1 (5%)	1	
Yes	53	16 (30%)	8.72 (1.16-65.79)	0.036

developed a local recurrence, where only 2 out of 32 (6%) developed a local recurrence in the non hypoxic group (p=0.004, Fig. 2d). Univariate Cox regression analysis predicted a significantly worse local control (HR 6.32, 95% CI 1.47-27.15) for patients with a hypoxic pattern.

Multivariate analysis

High expression of either HIF1α or CA-IX separately or combined was the best predictor for local recurrence after radiotherapy, independent of all other tested variables (HR 5.67, 95% CI 1.30-24.76) (Table 4).

Table 4. Patient characteristics related to local recurrence after radiotherapy (multivariate HR, 95% CI, p-value)

Characteristics	HR (95% CI)	P-Value
HIF1α + CA-IX expression		
Both high or combination high/low	5.67 (1.30-24.76)	**0.021**
Pre-treatment haemoglobin level		
Low	1.65 (0.68-4.03)	0.272

High HIF1α expression predicts a worse overall survival

Univariate Cox regression analysis showed that high HIF1α expression (HR 2.92, 95% CI 1.22-6.99) and age >66 (HR 3.36, 95% CI 1.46-7.76) were significantly associated with worse overall survival (OS). Low pre-treatment haemoglobin levels showed a trend towards worse overall survival (HR 2.14, 95% CI 0.97-4.73). Neither CA-IX, nor GLUT1 expression, or one of the other parameters showed a significant relation with overall survival (results not shown).

In a multivariate Cox regression analysis high HIF1α (HR 2.56, 1.07-6.15) and an age over 66 (HR 3.03, 95% CI 1.30-7.02) were significantly associated with worse OS (Table 5).

Table 5. Patient characteristics related to overall survival after radiotherapy (multivariate HR, 95% CI, p-value)

Characteristics	HR (95% CI)	P-Value
HIF1α		
High	2.56 (1.07-6.15)	**0.036**
Age		
>66 years	3.03 (1.30-7.02)	**0.010**

DISCUSSION

Surgery and radiotherapy are both well established and effective treatment modalities for early stage laryngeal carcinoma, with similar local control and survival rates, but with better functional results after radiotherapy. The choice of treatment is mainly dependent on geography, with a preference for surgery in the United States, and a preference for radiotherapy in the western part of Europe, including the Netherlands. Local recurrences occur in 5-25% of the laryngeal carcinomas treated with radiotherapy. The main purpose of the current study was to predict which cases have a high probability on developing local recurrence after radiotherapy as in these cases primary surgery or hypoxic modification might be a good alternative. We showed that high HIF1α and CA-IX expression are possibly significant prognostic factors for local recurrence in early stage glottic carcinomas which need to be validated in an independent dataset. More importantly, we identified a subgroup of glottic laryngeal carcinomas with a very high local control rate after radiotherapy, i.e. the cases with low expression of both HIF1α and CA-IX.

To measure hypoxia in tumours, several techniques are available, using polygrafic needle electrodes, extrinsic or intrinsic markers and by the use of PET scan[5,6,8]. A direct way of tumour oxygen measurement is via an invasive technique using polarographic needle electrodes. Measurement of tumour hypoxia using Eppendorf electrodes has been studied in various types of solid tumours, including

squamous cell head and neck cancer, and is also associated with worse loco-regional control and survival after radiotherapy[20,21]. Several studies have shown that tumour hypoxia measured by an electrode is associated with worse tumour control in HNSCC treated with radiotherapy[22]. Although this method is regarded as the gold standard, the main disadvantages of the Eppendorf method are its invasiveness and the fact that many tumours are not accessible with the electrode, as is the case in early stage glottic carcinoma. In the clinical setting, extrinsic tumour markers such as Pimonidazole and EF5 have been used[23,24]. However, no correlation was shown between extrinsic hypoxia markers and oxygenation status measured by electrodes, so the usefulness of extrinsic markers to measure hypoxia is questionable[25,26].

For measuring hypoxia in early stage glottic carcinoma intrinsic hypoxia markers may be more appropriate. The main advantage is that the intrinsic markers can be tested on the same pre-treatment biopsy samples used for histopathological classification. Studies on cervical cancer treated with radiotherapy show a good correlation between oxygenation status measured by Eppendorf electrode and the expression of the intrinsic hypoxia markers HIF1α and CA-IX[11,27]. For GLUT-1 the relation between oxygenation status and overexpression is less clear. Airley et al. found a weak correlation between oxygenation status and GLUT-1 overexpression (r=0.28) whereas a study by Mayer et al. failed to show a correlation between oxygenation status and GLUT-1 expression[10,28]. This indicates that GLUT-1 might not be a good intrinsic marker for tumour hypoxia. GLUT-1 expression is induced by other stimuli than HIF1α, as discussed by Mayer et al[28]. This is a possible explanation for the lack of correlation between HIF1α and GLUT-1 as found in the current study.

In HNSCC, results concerning the prognostic significance of immuno-histochemical staining of HIF1α, CA-IX and GLUT-1 are somewhat conflicting. Some studies showed an adverse relationship between overexpression of HIF1α, CA-IX and GLUT-1 and loco(regional) control[19,23,29], disease free survival[29,30], disease specific survival[30] and response to radiotherapy[29,31]. In other studies, no

significant relationship was found between overexpression of the intrinsic hypoxia markers and clinical outcome[12,19,23,32], or even showed an inverse relationship[33]. How can these apparent conflicting results be explained. Tumours arising in different head and neck sites are heterogeneous with regard to incidence and etiology, the use of different treatment strategies and biological and clinical behaviour. This heterogeneity may, at least partly, explain the differences regarding the prognostic significance of intrinsic tumour markers among the different studies. For this reason, we decided to focus on one well-defined subpopulation of T1/T2 glottic carcinoma treated with radiotherapy alone. The results in this specific subgroup of HNSCC showed that an adverse hypoxic, defined as overexpression of either HIF1α, CA-IX or both, was associated with worse local control, i.e. approximately 60% after 5 years. However, we did not find a significant association between GLUT-1 overexpression, local control and overall survival. A possible reason for this is the relatively small number of failures in our study (21 local recurrences and 26 deaths) only allowing detection of large differences between subgroups.

The question arises as to whether HIF1α and CA-IX could be used to select patients for alternative treatment modalities instead of primary radiotherapy, such as CO_2 laser surgery or partial laryngectomy. Furthermore, this group of patients potentially benefits from treatment with hypoxic modification as hyperbaric oxygen treatment or the use of hypoxic sensitizers as Nitromidazoles or ARCON (accelerated radiotherapy with carbogen and nicotinamide). At present, hypoxic modification is not widely used in the general practice. However, a recent survey performed by Overgaard showed that patients with HNSCC had significantly better local-regional control and overall survival when hypoxia modifiers were added to primary radiotherapy alone[34]. Hyperbaric oxygen treatment proved to be more effective than normobaric treatment or treatment with hypoxic sensitizers[34]. The additional value of ARCON versus accelerated radiotherapy alone is subject of investigation of a recently closed randomized controlled trial in the Netherlands.

Our findings can also have important clinical implications for late stage laryngeal carcinomas. For late stage glottic carcinoma, treatment options are radiotherapy alone, radiotherapy combined with chemotherapy, and primary surgery with or without postoperative radiotherapy. In head and neck cancer in general, combined assessment of radiotherapy with chemotherapy has been proven more efficient than radiotherapy alone[35]. In our center, for locally advanced glottic carcinoma, radiotherapy is preferred above surgery because of preservation of laryngeal function. Patients with a late stage glottic carcinoma showing a hypoxic profile potentially can benefit from other treatment options than radiotherapy alone, such as chemoradiation or primary surgery.

CONCLUSION

The results of the current study indicate that CA-IX and HIF1α are possibly significant prognostic markers for local control in a very homogenous study population consisting only of early stage glottic laryngeal carcinoma treated with radiotherapy alone.In this specific subset of patients, the addition of hypoxic modification should be considered. Future studies should clarify whether this subset of patients are better of with primary surgery.

REFERENCES

1. Nix P, Cawkwell L, Patmore H, Greenman J, Stafford N. Bcl-2 expression predicts radiotherapy failure in laryngeal cancer. *Br J Cancer*. 2005;92(12):2185-2189.
2. Jones AS, Fish B, Fenton JE, Husband DJ. The treatment of early laryngeal cancers (T1-T2 N0): Surgery or irradiation? *Head Neck*. 2004;26(1043-3074; 2):127-135.
3. Spector JG, Sessions DG, Chao KS, Hanson JM, Simpson JR, Perez CA. Management of stage II (T2N0M0) glottic carcinoma by radiotherapy and conservation surgery. *Head Neck*. 1999;21(1043-3074; 2):116-123.
4. Hoogsteen IJ, Marres HA, van der Kogel AJ, Kaanders JH. The hypoxic tumour microenvironment, patient selection and hypoxia-modifying treatments. *Clin Oncol (R Coll Radiol)*. 2007(0936-6555).
5. Vaupel P, Mayer A. Hypoxia in cancer: Significance and impact on clinical outcome. *Cancer Metastasis Rev*. 2007(0167-7659).
6. Vordermark D, Brown JM. Endogenous markers of tumor hypoxia predictors of clinical radiation resistance? *Strahlenther Onkol*. 2003;179(0179-7158; 12):801-811.
7. Hoogsteen IJ, Marres HA, Bussink J, van der Kogel AJ, Kaanders JH. Tumor microenvironment in head and neck squamous cell carcinomas: Predictive value and clinical relevance of hypoxic markers. A review. *Head Neck*. 2007;29(1043-3074; 6):591-604.
8. Bussink J, Kaanders JH, van der Kogel AJ. Tumor hypoxia at the micro-regional level: Clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. *Radiother Oncol*. 2003;67(0167-8140; 1):3-15.
9. Bos R, van der Groep P, Greijer AE, et al. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*. 2003;97(6):1573-1581.
10. Airley R, Loncaster J, Davidson S, et al. Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. *Clin Cancer Res*. 2001;7(4):928-934.
11. Loncaster JA, Harris AL, Davidson SE, et al. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: Correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res*. 2001;61(0008-5472; 17): 6394-6399.

12. Hui EP, Chan AT, Pezzella F, et al. Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. *Clin Cancer Res*. 2002;8(8):2595-2604.

13. Bache M, Reddemann R, Said HM, et al. Immunohistochemical detection of osteopontin in advanced head-and-neck cancer: Prognostic role and correlation with oxygen electrode measurements, hypoxia-inducible-factor-1alpha-related markers, and hemoglobin levels. *Int J Radiat Oncol Biol Phys*. 2006.

14. Rades D, Kuhn H, Schultze J, et al. Prognostic factors affecting locally recurrent rectal cancer and clinical significance of hemoglobin. *Int J Radiat Oncol Biol Phys*. 2007(0360-3016).

15. Pastorekova S, Zavadova Z, Kostal M, Babusikova O, Zavada J. A novel quasi-viral agent, MaTu, is a two-component system. *Virology*. 1992;187(0042-6822; 2):620-626.

16. Schutter HD, Barbe B, Spaepen M, et al. Microsatellite alterations in head and neck squamous cell carcinoma and relation to expression of pimonidazole, CA IX and GLUT-1. *Radiother Oncol*. 2006.

17. Metz CE. Basic principles of ROC analysis. *Semin Nucl Med*. 1978;8(0001-2998; 4):283-298.

18. Peduzzi P, Concato J, Feinstein AR, Holford TR. Importance of events per independent variable in proportional hazards regression analysis. II. accuracy and precision of regression estimates. *J Clin Epidemiol*. 1995;48(0895-4356; 12):1503-1510.

19. Nordsmark M, Eriksen JG, Gebski V, Alsner J, Horsman MR, Overgaard J. Differential risk assessments from five hypoxia specific assays: The basis for biologically adapted individualized radiotherapy in advanced head and neck cancer patients. *Radiother Oncol*. 2007;83(0167-8140; 3):389-397.

20. Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhirst MW. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys*. 1997;38(0360-3016; 2):285-289.

21. Nordsmark M, Bentzen SM, Rudat V, et al. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. an international multi-center study. *Radiother Oncol*. 2005;77(1):18-24.

22. Nordsmark M, Overgaard J. A confirmatory prognostic study on oxygenation status and loco-regional control in advanced head and neck squamous cell carcinoma treated by radiation therapy. *Radiother Oncol*. 2000;57(1):39-43.

23. Kaanders JH, Wijffels KI, Marres HA, et al. Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. *Cancer Res*. 2002;62(23):7066-7074.

24. Evans SM, Hahn S, Pook DR, et al. Detection of hypoxia in human squamous cell carcinoma by EF5 binding. *Cancer Res*. 2000;60(0008-5472; 7):2018-2024.

25. Evans SM, Judy KD, Dunphy I, et al. Comparative measurements of hypoxia in human brain tumors using needle electrodes and EF5 binding. *Cancer Res*. 2004;64(0008-5472; 5): 1886-1892.

26. Nordsmark M, Loncaster J, quino-Parsons C, et al. Measurements of hypoxia using pimonidazole and polarographic oxygen-sensitive electrodes in human cervix carcinomas. *Radiother Oncol*. 2003;67(0167-8140; 1):35-44.

27. Haugland HK, Vukovic V, Pintilie M, et al. Expression of hypoxia-inducible factor-1alpha in cervical carcinomas: Correlation with tumor oxygenation. *Int J Radiat Oncol Biol Phys*. 2002;53(0360-3016; 4):854-861.

28. Mayer A, Hockel M, Wree A, Vaupel P. Microregional expression of glucose transporter-1 and oxygenation status: Lack of correlation in locally advanced cervical cancers. *Clin Cancer Res*. 2005;11(1078-0432; 7):2768-2773.

29. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. Hypoxia-inducible factor (HIF1α and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int J Radiat Oncol Biol Phys*. 2002;53(5):1192-1202.

30. Winter SC, Shah KA, Han C, et al. The relation between hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression with anemia and outcome in surgically treated head and neck cancer. *Cancer*. 2006;107(4):757-766.

31. Kunkel M, Moergel M, Stockinger M, et al. Overexpression of GLUT-1 is associated with resistance to radiotherapy and adverse prognosis in squamous cell carcinoma of the oral cavity. *Oral Oncol*. 2007(1368-8375).

32. Eriksen JG, Overgaard J. Lack of prognostic and predictive value of CA IX in radiotherapy of squamous cell carcinoma of the head and neck with known modifiable hypoxia: An evaluation of the DAHANCA 5 study. *Radiother Oncol*. 2007;83(0167-8140; 3):383-388.

33. Beasley NJ, Leek R, Alam M, et al. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: Relationship to tumor biology and treatment outcome in surgically resected patients. *Cancer Res*. 2002;62(9):2493-2497.

34. Overgaard J. Hypoxic radiosensitization: Adored and ignored. *J Clin Oncol*. 2007;25(1527-7755; 26):4066-4074.

35. Forastiere AA, Pajak TF, Maor M, Weber R. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer (vol 349, pg 2091, 2003). *N Engl J Med*. 2004;350(10):1052-1053.

Chapter 6

EGFR overexpression is associated with lymph node positivity and supraglottic location in T1/T2 laryngeal squamous cell carcinoma treated with radiotherapy

Submitted

Michiel Schrijvers
Jasper Wachters
Lorian Slagter-Menkema
Mirjam Mastik
Leonie Bruine de Bruin
Johannes Langendijk
Truuske de Bock
Jacqueline van der Wal
Ed Schuuring
Bernard van der Laan

ABSTRACT

PURPOSE The aim of this study was to establish the prognostic effect of the epidermal growth factor receptor (EGFR) expression on local control, survival and lymph node metastasis in patients with laryngeal carcinoma treated with radiotherapy only.

METHODS AND MATERIALS Immunohistochemical staining for EGFR was performed on pre-treatment biopsies of 139 patients with T1-T2 laryngeal squamous cell carcinoma primarily treated with radiotherapy between 1997 and 2008. Pearson's correlation test, Kaplan Meier analysis and Cox regression analysis was performed to correlate expression levels with local control, overall survival, disease specific survival and lymph node status.

RESULTS EGFR overexpression was significantly associated with positive N status and supraglottic tumour location (Odds ratio (OR) 8.71, 95 % confidence interval (CI) 1.12-67.93, p=0.039 and OR 14.56, 95% CI 4.22-50.28, p=0.000 respectively). Kaplan Meier survival analysis and Cox regression analysis did not show a significant relation between EGFR expression and local control (HR 1.19, 95% CI 0.58-2.42, p=0.64), overall survival (HR 0.62, 95% CI 0.31-1.22, p=0.16) and disease specific survival (HR 1.19, 95% 0.33-4.34, p=0.79).

CONCLUSIONS This study showed no relation between EGFR overexpression and local control, overall survival and disease specific survival in a group of mainly early stage laryngeal carcinoma. We did found a significant association between EGFR overexpression and supraglottic tumour location and lymph node positivity. These groups might benefit from adding an EGFR blocking agent to the accelerated RT.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous group of tumours arising in the oral cavity, oropharynx, hypopharynx and larynx. The annual incidence is approximately 600.000 worldwide, with a 5 year survival rate of 40-50%[1]. Treatment for locally advanced tumours consists of a combination of surgery, radiotherapy and/or chemotherapy. Early stage tumours are normally treated by single modalities, either radiotherapy or surgery. Of all HNSCC, 20% arises in the larynx. In this group, the majority are located in the glottic region. Most early stage (T1/T2) laryngeal squamous cell carcinomas (LSCC) are treated with radiotherapy only. Selection of patients for radiotherapy is predominantly based on T and N stage. The 5-year local control rate for T1 LSCC varies between 85% and 94%, and for T2 LSCC between 70% and 80%[2], and until now it remains difficult to predict clinical outcome. A possible explanation for different outcome in early stage tumours treated with similar radiotherapy schedules are specific molecular or cell biological tumour markers associated with radioresistance. One of the possible prognostic markers is the epidermal growth factor receptor (EGFR)[3]

EGFR is a transmembrane glycoprotein consisting of an extracellular ligand binding domain, a transmembrane region, and an intracellular tyrosine kinase domain[4,5]. Upon binding of a specific ligand, phosphorylation of the intracellular tyrosine kinase occurs, activating the RAS/MAPK and PI3K/PTEN/AKT pathways. This results in numerous cellular responses, such as cell proliferation, insensitivity to apoptosis, migration and differentiation[6,7].

In different cancer types including breast, lung, pancreatic, vulva, and cervical carcinoma, EGFR overexpression has been associated with worse local control, overall survival, disease specific survival and advanced lymph node metastasis[8,9]. More than 80% of the HNSCC show EGFR overexpression[10]. It has been suggested that EGFR plays an important role in resistance to radiotherapy, resulting in a decreased local control[3]

The prognostic value of EGFR overexpression on clinical outcome in HNSCC treated with radiotherapy has been studied by numerous groups, leading to conflicting results. In some studies EGFR overexpression was associated with worse local control and overall survival[11-15]. Other studies in HNSCC did not show a correlation between EGFR overexpression and clinical outcome[16-25]. It is difficult to compare these different studies because of heterogeneity in the use of different antibodies, treatment types, scoring evaluation, number of patients, tumour stage and tumour subsites.

Only few studies have been published on the prognostic value of EGFR-expression in laryngeal carcinoma treated with radiotherapy alone. Demiral et al. reported worse local control (LC) in a small group of 31 glottic carcinomas overexpressing EGFR[13]. Next to the relationship with locoregional control and survival, EGFR overexpression is associated with lymph node metastasis[26,27,11]. This is important since the presence of lymph node metastases is considered a reliable and consistent prognostic factor of regional recurrence and survival[28]. Since most studies included patients with different subsides, including the oral cavity, oropharynx, hypopharynx, and larynx, and therefore are difficult to compare, we constructed a homogenous group consisting of 139 T1/T2 glottic and supraglottic carcinomas treated with radiotherapy only.

The aim of this study was to establish the prognostic value of EGFR expression on local control, survival and lymph node metastasis in pre-treatment biopsies from a well-defined subset of LSCC.

MATERIALS AND METHODS

Patients and tissues

The study population of the present study was composed of patients with histologically confirmed squamous cell carcinoma of the glottis and supraglottis. The cohort of patients with glottic carcinoma was described in detail previously[29]. Between 1997 and 2008, demographic and clinicopathological data such as gender,

age, pre-treatment haemoglobin level, T-status, N-status of patients with laryngeal squamous cell carcinoma in the Northern part of the Netherlands (comprising >10 medical centres) and treated at our institute, the Isala Clinics Zwolle or the Radiotherapy Institute Friesland, were retrospectively collected by reviewing the patient charts. The inclusion criteria for this study were (1) histologically proven squamous cell carcinoma; (2) localized in the glottis or supraglottis; (3) cT1 and cT2; (4) no evidence for distant metastasis (cM0); (5) curatively treated with radiotherapy alone, and (6) no other previous or concurrent treatment modalities. Of all patients that fulfilled the inclusion criteria, formalin-fixed, paraffin-embedded pre-treatment biopsies taken at our institute were collected and revised by an experienced pathologist. Tissue specimens with sufficient tumour cells for immunohistochemical staining were available from 139 patients. The pre-treatment characteristics are summarized in Table 1. Informed consent was given by all patients included in the study.

Radiotherapy and follow-up

In all patients treated in the UMCG, radiotherapy was delivered with megavoltage equipment using 6 MV photons as reported previously[29]. T1 tumours were treated with a total dose of 66 Gy using 2 Gy per fraction, 5 times per week. T2 tumours were generally treated with 6 fractions per week to a total dose of 70 Gy in 6 weeks. In case of elective irradiation of the neck nodes, a total dose of 46 Gy was given on the primary PTV with an additional boost of 70 Gy on the primary tumour and pathological lymph nodes. After completing radiation, patients were followed every 3 months the first and second year, and every 6 months the third, fourth and fifth year. After five years without evidence of disease, patients were discharged from follow-up. In all patients, planning-CT scan was made in supine position. The target volumes were delineated as described in previous reports[30]. All patients were treated with 3D-conformal radiotherapy.

Table 1. Patient characteristics: laryngeal carcinoma treated with primarily radiotherapy

Characteristics	Glottis + Supraglottis N=139	Glottis N=87	Supraglottis N=52	P-value (X^2)
Sex				
Male	118 (85%)	77 (89%)	41 (79%)	0.146
Female	21 (15%)	10 (11%)	11 (21%)	
Age (years)				
Median (Range)	64 (33-96)	66 (40-86)	63 (33-96)	0.082
Primary symptom				
Hoarse voice	110 (79%)	83 (95%)	27 (52%)	
Swallowing disorder	5 (4%)	0 (0%)	10 (20%)	**0.000**
Other	24 (17%)	4 (5%)	15 (30%)	
T-status				
1	60 (43%)	45 (52%)	15 (29%)	**0.013**
2	79 (57%)	42 (48%)	37 (71%)	
N-status				
0	122 (88%)	84 (97%)	38 (73%)	**0.000**
1	17 (12%)	3 (3%)	14 (27%)	
Tumour Location				
Glottis	87 (63%)			
Supraglottis	52 (37%)			
EGFR Level				
High EGFR	95 (68%)	46 (53%)	49 (94%)	**0.000**
Low EGFR	44 (32%)	41 (47%)	3 (6%)	

Immunohistochemistry

EGFR protein in the tissue section was detected using the EGFR clone 113 (Novocastra, Newcastle upon Tyne, UK), according to the instructions of the manufacturer. EGFR staining was performed on 4 μm paraffin sections. Slides were deparaffinized in xylene twice for 10 minutes and subsequently rehydrated through a series of decreasing ethanol dilutions and PBS. Antigen retrieval was achieved by heating in a microwave in pre-heated Tris-HCL buffer. To block

endogenous peroxidase activity, H_2O_2 0.3% was applied for 30 minutes at room temperature. Slides were washed 3 times with PBS afterwards.

Slides were stained for 1 hour with the antibody against EGFR (Clone 113, 1:100). Secondary antibodies RAM^{PO} (Dakocytomation, Glostrup, Denmark) was diluted 1:100 in 1% BSA/PBS complemented with 1% human AB serum and applied for 30 minutes at room temperature. Tertiary antibody GAR^{PO} complex 1:100 was applied for 30 minutes at room temperature. The peroxidase reaction was performed by applying 3, 3' diaminobenzide tetrachlodride (DAB) for 10 minutes and after washing with PBS the slides were counterstained for 2 minutes with haematoxylin and fixated.

Scoring method

After having set the scoring method with an experienced pathologist, all slides were evaluated by 2 teams separately who were both blinded to clinical outcome. The discordant cases were reviewed by all observers and scores were reassigned on consensus of opinion. For EGFR, staining intensity was semi-quantitatively scored as - (negative staining), + (weak positive), ++ (positive) or +++ (strongly positive) as described previously[7] (Fig. 1). For statistical analysis, any positive staining above background was considered as high (+, ++ and +++).

Statistical analysis

All statistical analysis was performed using the statistical package SPSS 18.0.0 (SPSS, inc., Chicago, IL, USA). Associations between EGFR expression and the other pre-treatment parameters were analysed using a chi-square test. Follow-up time was calculated from the day of diagnosis until the date of the last follow-up. Local recurrence was defined as tumour recurrence at the primary tumour site, and was calculated from the date of diagnosis until the day of local recurrence or lost to follow-up. Overall survival was defined as the day of diagnosis until the day of death or lost to follow-up. Disease specific survival was defined as the day of diagnosis until the day of death of laryngeal cancer or lost to follow-up.

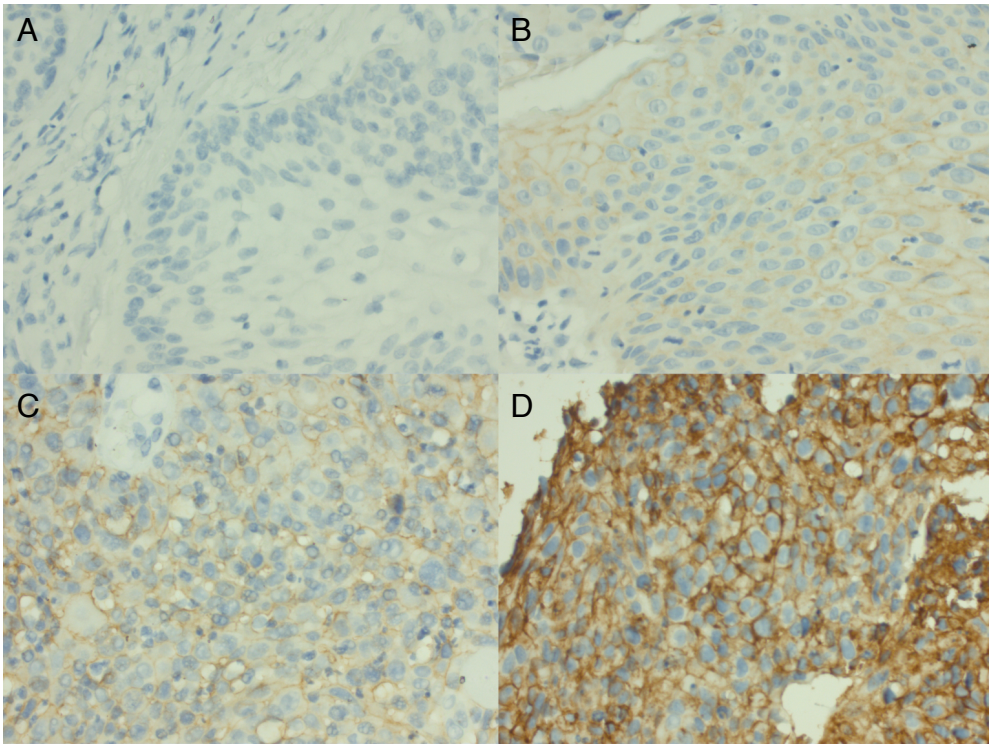

Figure 1. Immunohistochemical staining for EGFR, magnification 400x. Examples of negative cytoplasmic EGFR (A), weak positive (+) (B), positive (++) (C) and strongly positive (+++) (D).

Kaplan-Meier survival analysis, logistic regression and Cox regression analysis for the variable EGFR as well as for sex, age, T-status and N-status, was performed with local control (LC), overall survival (OS) and disease specific survival (DSS) as endpoints. P-values <0.05 were considered significant.

Table 2. Patient characteristics: follow-up

Characteristics	Glottis + Supraglottis N=139	Glottis N=87	Supraglottis N=52
Events in follow-up			
Local recurrence	36 (26%)	23 (26%)	13 (25%)
Regional rec.	4 (3%)	3 (3%)	1 (2%)
Death	47 (34%)	24 (27%)	23 (44%)
DOD	15 (33%)	7 (32%)	8 (35%)
DNOD	30 (67%)	15 (68%)	15 (65%)
Time to follow-up (months)			
Median (Range)	41 (1-166)	41 (1-119)	42 (5-166)
Time to local recurrence (months)			
Median (Range)	12 (2-46)	12 (2-46)	13 (5-18)
Time to regional recurrence (months)			
Median (Range)	16 (6-57)	13 (6-27)	57 (-)
Time to death (months)			
Median (Range)	29 (3-154)	29 (3-98)	31 (6-154)

RESULTS

Patient follow-up data

Thirty-six patients (26%) developed a local recurrence after radiotherapy. Of these patients, 31 were treated with a total laryngectomy. Five patients received palliative treatment. Two patients had a distant metastasis of the lung, in two patients the recurrence was considered unresectable, and one patient developed a second primary tumour of the lung with brain metastasis. Of all patients treated with a salvage laryngectomy, two patients developed a second locoregional recurrence. These two patients received palliative therapy. Forty-seven patients died, of which 15 of their index tumour (33%). Follow-up data are presented in Table 2. Comparison of the glottic and supraglottic group showed significant differences in primary symptoms, T-status, N-status and EGFR level (X^2 test , table 1).

EGFR overexpression is associated with positive lymph node status and supraglottic tumour location

In the total population (N=139), ninety-five patients (68%) showed high EGFR expression. In the glottic group 53% (46/87) and in the supraglottic group 94% (49/52) showed high EGFR expression. Logistic regression revealed a significant association between high EGFR expression and positive N status. (Odds ratio (OR) 8.71, 95% confidence interval (CI) 1.12-67.93, p=0.039) 94% in the N+ group showed EGFR overexpression (16/17) in comparison to 65% in the N0 group (79/122). High EGFR expression showed a significant association with supraglottic tumour location (OR 14.56, 95% CI 4.22-50.28, p=0.000). Multivariate logistic regression showed that supraglottic tumour location was mostly associated with high EGFR expression (OR 12.19, 95% CI 3.46-42.98, p=0.000).

No relation between EGFR expression local control and overall survival

Kaplan Meier survival analysis and Cox regression analysis did not show a significant relation between EGFR expression and local control in our series of 139 laryngeal carcinomas (Fig. 2, Hazard ratio (HR) 1.19, 95% CI 0.58-2.42, p=0.64). No relation was found between EGFR overexpression with neither overall survival (HR 0.62, 95% CI 0.31-1.22, p=0.16) or disease specific survival, respectively (HR 1.19, 95% 0.33-4.34, p=0.79. In the supraglottic group (n=52), Kaplan Meier analysis suggested a relation between EGFR overexpression and worse local control, but the number of cases included were to low to reach significance (Fig. 3, p=0.375).

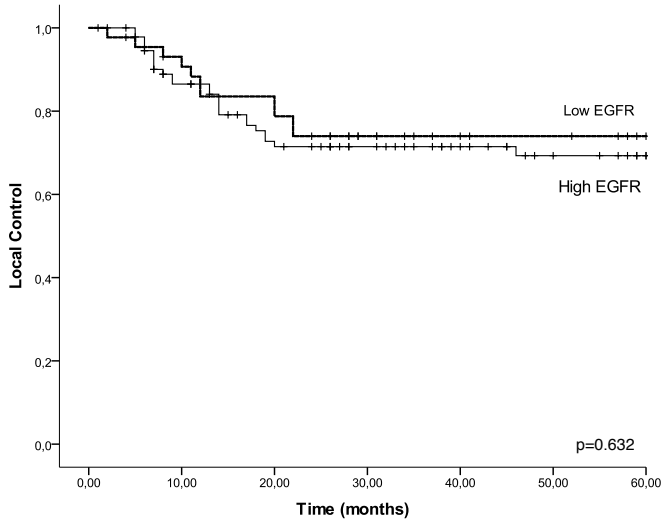

Figure 2. Local control as a function of EGFR expression (glottic and supraglottic carcinoma, N=139)

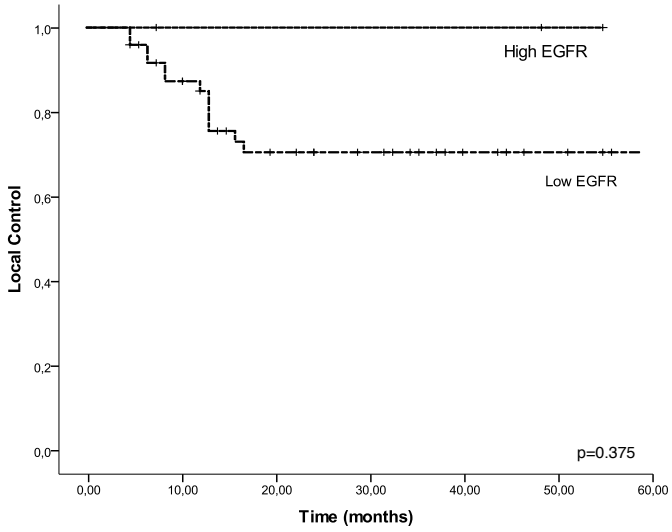

Figure 3. Local control as a function of EGFR expression in the supraglottic carcinoma group (n=52)

DISCUSSION

We showed a significant association between high EGFR expression and positive lymph node status. Furthermore, we showed a positive association between high EGFR expression and tumour location. Significantly more tumours in the supraglottic group showed EGFR overexpression compared to the glottic group (94% vs. 53%). The association between high EGFR expression and positive lymph node status is in agreement with its biological function since activation of EGFR signaling pathways is reported to lead to increased proliferation, metastasis, angiogenesis and decreased apoptosis in tumour cells[31,32]. Furthermore, in a recent study, Sarkis et al. reported a positive correlation between EGFR overexpression and D2-40 (a new immunohistochemical marker for lymphatic endothelium) positive lymphatic vessels, suggesting a higher tendency for lymphatic dissemination in a group of oral squamous cell carcinomas[33].

For early stage laryngeal carcinoma, radiotherapy as a single treatment modality is often the treatment of first choice. In our institution, only T1a glottic carcinomas with normal/diminished mucosal wave are treated with Co2 laser surgery[34]. Although radiotherapy is a good treatment option, recurrences occur in 6-28% of the cases[2].

We studied a homogenous group consisting of laryngeal carcinomas treated with radiotherapy only. Since it has been suggested that EGFR plays an important role in resistance to radiotherapy, it would be interesting to know whether there is an effect of EGFR expression on the clinical outcome after radiotherapy[3,35-37]. One of the possible reasons for radioresistance in HNSCC is accelerated repopulation of tumour cells after exposure to ionizing radiation[3]. In a preclinical study, Dent et al showed that irradiation could lead to activation of EGFR associated with increased proliferation and increased repopulation during radiotherapy[38]. Therefore, an increase in EGFR induced by irradiation may lead to increased radioresistance in HNSCC. One possibility to reduce the rapid repopulation of tumour cells during radiotherapy is to treat patients with accelerated radiotherapy. Indeed, two studies

showed that local control in patients overexpressing EGFR was better when treated with accelerated RTH compared to conventional RTH[39,16] . For several years now, after the publication of a number of randomized controlled trials, accelerated radiotherapy is increasingly used as the new standard in our institution for all laryngeal carcinoma, including those with T1-T2 tumours[40,41]. In a previous report, we showed that these patients had better LC compared to those treated with conventional RT (Chapter 3). In our study population, the patients were treated with curative radiotherapy in 3 different centers in the Northern part of the Netherlands. Furthermore, before 2000, most patients in our institute were treated with conventional fractionation schedules. Therefore, as patients in the present cohort have been treated with different fractionation schedules, the added value of accelerated fractionation among patients with high EGFR expression cannot be determined.

In many tumour types, high EGFR expression is associated with worse local control and overall survival after surgery or chemoradiotherapy[11,42-44]. In HNSCC, conflicting results have been published. Ang et al. reported worse LC, OS and DOD in a group of 155 advanced HNSCC's treated with radiation therapy overexpressing EGFR[11]. Eriksen et al. failed to show a significant relationship between EGFR overexpression and LC in a group of 336 HNSCC's treated with conventional or accelerated radiation therapy[39], which is in agreement with the results of the current study, in which no significant relationship was found between EGFR expression and neither local control, overall survival nor disease specific survival in a homogenous group consisting of laryngeal carcinoma treated with radiotherapy alone. We only found a borderline significant relationship between high EGFR expression and worse LC in the subset of T2 supraglottic tumours. The interpretation of the results in this subset are however difficult since there were only 3 cases with low EGFR expression.

There are many possible reasons for conflicting results between different studies. First, the prognostic significance of EGFR has been investigated in heterogeneous populations treated with different treatment modalities. Second,

there is no general consensus regarding the staining protocols and scoring methods[7].

Besides its role in resistance to radiotherapy and its possible role as prognostic marker, EGFR is an interesting subject for investigation because it can be targeted with EGFR blocking agents. To block EGFR two strategies have been developed. The first strategy targets the intracellular domain of the receptor by blocking the ATP binding site in the tyrosine kinase domain of EGFR by the use of tyrosine kinase inhibitors gefitinib and erlotinib[45]. This inhibits downstream signalling pathways, resulting in decreased cell proliferation and survival. The second strategy targets the extracellular domain of the EGFR. The most commonly used agent is the monoclonal antibody cetuximab. The binding of cetuximab to the EGFR leads to internalization and degradation of the antibody-receptor complex, downregulating EGFR expression[3]. In SCC cell lines Bonner showed that the combination of cetuximab and radiation resulted in decreased cellular proliferation[46].

Recently, Bonner et al. showed that among patients with stage III-IV HNSCC, the addition of cetuximab to radiotherapy resulted in a significant improvement of locoregional control and survival as compared to radiotherapy alone, without enhancing radiation-induced toxicity[47,48]. Based on the results of this study, cetuximab is now increasingly used in combination with definitive radiotherapy in locally advanced disease in particular for patients in which concurrent chemoradiation is considered not feasible. So far, no studies have been published which compared concomitant cetuximab and radiation therapy and/or in combination with EGFR levels in early stage laryngeal carcinoma.

In our study population, EGFR expression was not associated with local control or overall survival. We did show a significant correlation between high EGFR expression and positive lymph node status and supraglottic tumour location. It is well accepted that a positive lymph node status has an adverse effect on regional control and survival[49,50]. Since 94% of the supraglottic tumours and 90% of the patients with positive lymph node status showed pre-treatment overexpression of

EGFR, these groups also might benefit from adding an EGFR blocking agent to the accelerated RT.

CONCLUSION

We did not find an association between EGFR overexpression and local control in a homogeneous well-defined group of laryngeal carcinoma primarily treated with radiotherapy only. Interestingly, high expression was more prominent in the supraglottic versus glottis group confirming that these two laryngeal localizations may represent different biological and clinical entities. Furthermore, EGFR overexpression was associated with lymph node positivity. Possibly, lymph-node-positive supraglottic carcinomas will benefit from accelerated RT combined with EGFR-targeted therapy.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin*. 2010;60(1542-4863; 0007-9235; 5):277-300.
2. Mendenhall WM, Amdur RJ, Morris CG, Hinerman RW. T1-T2N0 squamous cell carcinoma of the glottic larynx treated with radiation therapy. *J Clin Oncol*. 2001;19(0732-183; 20): 4029-4036.
3. Zimmermann M, Zouhair A, Azria D, Ozsahin M. The epidermal growth factor receptor (EGFR) in head and neck cancer: Its role and treatment implications. *Radiat Oncol*. 2006;1:11.
4. Milas L, Mason K, Hunter N, et al. In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin Cancer Res*. 2000;6(1078-0432; 2):701-708.
5. Chung KY, Shia J, Kemeny NE, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol*. 2005;23(0732-183; 9):1803-1810.
6. Herbst RS. Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys*. 2004;59(2 Suppl):21-26.
7. Pattje WJ, Schuuring E, Mastik MF, et al. The phosphatase and tensin homologue deleted on chromosome 10 mediates radiosensitivity in head and neck cancer. *Br J Cancer*. 2010;102(1532-1827; 0007-0920; 12):1778-1785.
8. Hirsch FR, Varella-Garcia M, Cappuzzo F. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene*. 2009;28 Suppl 1:S32-7.
9. Woelber L, Hess S, Bohlken H, et al. EGFR gene copy number increase in vulvar carcinomas is linked with poor clinical outcome. *J Clin Pathol*. 2012;65(2):133-139.
10. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res*. 1993;53(0008-5472; 15):3579-3584.
11. Ang KK, Berkey BA, Tu X, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res*. 2002;62(0008-5472; 24):7350-7356.
12. Chang AR, Wu HG, Park CI, Jun YK, Kim CW. Expression of epidermal growth factor receptor and cyclin D1 in pretreatment biopsies as a predictive factor of radiotherapy efficacy in early glottic cancer. *Head Neck*. 2008;30(1097-0347; 1043-3074; 7):852-857.

13. Demiral AN, Sarioglu S, Birlik B, Sen M, Kinay M. Prognostic significance of EGF receptor expression in early glottic cancer. *Auris Nasus Larynx*. 2004;31(0385-8146; 4):417-424.

14. Hofman P, Butori C, Havet K, et al. Prognostic significance of cortactin levels in head and neck squamous cell carcinoma: Comparison with epidermal growth factor receptor status. *Br J Cancer*. 2008;98(0007-0920; 5):956-964.

15. Laimer K, Spizzo G, Gastl G, et al. High EGFR expression predicts poor prognosis in patients with squamous cell carcinoma of the oral cavity and oropharynx: A TMA-based immunohistochemical analysis. *Oral Oncol*. 2007;43(2):193-198.

16. Bentzen SM, Atasoy BM, Daley FM, et al. Epidermal growth factor receptor expression in pretreatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. *J Clin Oncol*. 2005;23(0732-183; 24):5560-5567.

17. Carracedo DG, Astudillo A, Rodrigo JP, Suarez C, Gonzalez MV. Skp2, p27kip1 and EGFR assessment in head and neck squamous cell carcinoma: Prognostic implications. *Oncol Rep*. 2008;20(3):589-595.

18. Eriksen JG, Steiniche T, Overgaard J. The role of epidermal growth factor receptor and E-cadherin for the outcome of reduction in the overall treatment time of radiotherapy of supraglottic larynx squamous cell carcinoma. *Acta Oncol*. 2005;44(1):50-58.

19. Fischer C, Zlobec I, Stockli E, et al. Is immunohistochemical epidermal growth factor receptor expression overestimated as a prognostic factor in head-neck squamous cell carcinoma? A retrospective analysis based on a tissue microarray of 365 carcinomas. *Hum Pathol*. 2008;39(10):1527-1534.

20. Gupta AK, McKenna WG, Weber CN, et al. Local recurrence in head and neck cancer: Relationship to radiation resistance and signal transduction. *Clin Cancer Res*. 2002;8(3): 885-892.

21. Preuss SF, Weinell A, Molitor M, et al. Survivin and epidermal growth factor receptor expression in surgically treated oropharyngeal squamous cell carcinoma. *Head Neck*. 2008;30(10): 1318-1324.

22. Ryott M, Wangsa D, Heselmeyer-Haddad K, et al. EGFR protein overexpression and gene copy number increases in oral tongue squamous cell carcinoma. *Eur J Cancer*. 2009;45(9):1700-1708.

23. Sheikh Ali MA, Gunduz M, Nagatsuka H, et al. Expression and mutation analysis of epidermal growth factor receptor in head and neck squamous cell carcinoma. *Cancer Sci*. 2008;99(8): 1589-1594.

24. Smid EJ, Stoter TR, Bloemena E, et al. The importance of immunohistochemical expression of EGFr in squamous cell carcinoma of the oral cavity treated with surgery and postoperative radiotherapy. *Int J Radiat Oncol Biol Phys*. 2006;65(5):1323-1329.

25. Pivot X, Magne N, Guardiola E, et al. Prognostic impact of the epidermal growth factor receptor levels for patients with larynx and hypopharynx cancer. *Oral Oncol*. 2005;41(3):320-327.

26. Rubin Grandis J, Melhem MF, Gooding WE, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst*. 1998;90(11): 824-832.

27. Xia W, Lau YK, Zhang HZ, et al. Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. *Clin Cancer Res*. 1999;5(12):4164-4174.

28. Michikawa C, Uzawa N, Sato H, Ohyama Y, Okada N, Amagasa T. Epidermal growth factor receptor gene copy number aberration at the primary tumour is significantly associated with extracapsular spread in oral cancer. *Br J Cancer*. 2011;104(5):850-855.

29. Schrijvers ML, van der Laan BF, de Bock GH, et al. Overexpression of intrinsic hypoxia markers HIF1alpha and CA-IX predict for local recurrence in stage T1-T2 glottic laryngeal carcinoma treated with radiotherapy. *Int J Radiat Oncol Biol Phys*. 2008;72(0360-3016; 0360-3016; 1): 161-169.

30. Beetz I, Schilstra C, Burlage FR, et al. Development of NTCP models for head and neck cancer patients treated with three-dimensional conformal radiotherapy for xerostomia and sticky saliva: The role of dosimetric and clinical factors. *Radiother Oncol*. 2011.

31. Baba Y, Fujii M, Tokumaru Y, Kato Y. Present and future of EGFR inhibitors for head and neck squamous cell cancer. *J Oncol*. 2012;2012:986725.

32. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.

33. Sarkis SA, Abdullah BH, Abdul Majeed BA, Talabani NG. Immunohistochemical expression of epidermal growth factor receptor (EGFR) in oral squamous cell carcinoma in relation to proliferation, apoptosis, angiogenesis and lymphangiogenesis. *Head Neck Oncol*. 2010;2:13.

34. Schrijvers ML, van Riel EL, Langendijk JA, et al. Higher laryngeal preservation rate after CO2 laser surgery compared with radiotherapy in T1a glottic laryngeal carcinoma. *Head Neck*. 2009;31(1097-0347; 1043-3074; 6):759-764.

35. Krause M, Zips D, Thames HD, Kummermehr J, Baumann M. Preclinical evaluation of molecular-targeted anticancer agents for radiotherapy. *Radiother Oncol*. 2006;80(2):112-122.

36. Krause M, Prager J, Zhou X, et al. EGFR-TK inhibition before radiotherapy reduces tumour volume but does not improve local control: Differential response of cancer stem cells and nontumourigenic cells? *Radiother Oncol*. 2007;83(3):316-325.

37. Normanno N, De Luca A, Bianco C, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene*. 2006;366(1):2-16.

38. Dent P, Reardon DB, Park JS, et al. Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. *Mol Biol Cell*. 1999;10(8):2493-2506.

39. Eriksen JG, Buffa FM, Alsner J, Steiniche T, Bentzen SM, Overgaard J. Molecular profiles as predictive marker for the effect of overall treatment time of radiotherapy in supraglottic larynx squamous cell carcinomas. *Radiother Oncol*. 2004;72(3):275-282.

40. Fu KK, Pajak TF, Trotti A, et al. A radiation therapy oncology group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: First report of RTOG 9003. *Int J Radiat Oncol Biol Phys*. 2000;48(0360-3016; 1):7-16.

41. Overgaard J, Hansen HS, Specht L, et al. Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6 and 7 randomised controlled trial. *Lancet*. 2003;362(1474-547; 9388):933-940.

42. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37 Suppl 4(0959-8049; 0959-8049):S9-15.

43. Pivot X, Magne N, Guardiola E, et al. Prognostic impact of the epidermal growth factor receptor levels for patients with larynx and hypopharynx cancer. *Oral Oncol*. 2005;41(3):320-327.

44. Galizia G, Lieto E, Ferraraccio F, et al. Prognostic significance of epidermal growth factor receptor expression in colon cancer patients undergoing curative surgery. *Ann Surg Oncol*. 2006;13(1068-9265; 1068-9265; 6):823-835.

45. Cohen EE, Kane MA, List MA, et al. Phase II trial of gefitinib 250 mg daily in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. *Clin Cancer Res*. 2005;11(23):8418-8424.

46. Bonner JA, Raisch KP, Trummell HQ, et al. Enhanced apoptosis with combination C225/radiation treatment serves as the impetus for clinical investigation in head and neck cancers. *J Clin Oncol*. 2000;18(21 Suppl):47S-53S.

47. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006;354(1533-4406; 6):567-578.

48. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol*. 2010;11(1):21-28.

49. Grandi C, Alloisio M, Moglia D, et al. Prognostic significance of lymphatic spread in head and neck carcinomas: Therapeutic implications. *Head Neck Surg*. 1985;8(2):67-73.

50. Snow GB, Annyas AA, van Slooten EA, Bartelink H, Hart AA. Prognostic factors of neck node metastasis. *Clin Otolaryngol Allied Sci*. 1982;7(3):185-192.

Chapter 7

General discussion and conclusion

DISCUSSION AND CONCLUSION

In the last decades, in HNSCC many tumour markers have been studied such as clinical and molecular markers to predict clinical outcome after therapy and to find targets for therapeutic intervention. In this thesis, we focused on early stage laryngeal carcinoma (LSCC) treated with radiotherapy only. We investigated several potential biomarkers which can predict clinical outcome.

Fass associated death domain (FADD)

In a previous study, Gibcus and coworkers reported that the frequently amplified chromosome 11q13 region contains multiple genes of which FADD was not only amplified the most in laryngeal/pharyngeal carcinoma, but overexpression and amplification of FADD also correlated with increased FADD protein expression[1]. We demonstrated that overexpression of FADD and its phosphorylated isoform pFADD is associated with increased local control in early stage glottic carcinoma treated with radiotherapy (**chapter 4**). Two other genes located in the 11q13 amplicon, cyclin D1 and cortactin have been suggested as potential biomarkers in HNSCC, but in our series of LSCC both these genes were not associated with local control. Cyclin D1 was reported to be involved in the transition from G1 to the S phase of the cell cycle[2]. Our results are in agreement with a study in laryngeal carcinoma that failed to associate cyclin D1 expression and clinical outcome[3]. Cortactin was associated with cell migration and invasion in vitro[4]. Since we studied early stage (T1/T2) glottic carcinomas with very few cases with lymph node metastasis (see table 1 in chapter 4), the lack of association between cortactin and local control is in good agreement with its biological function.

Our data suggest that the increased expression of pFADD might mediate the sensitivity of tumour cells to radiotherapy. Besides the role of FADD in apoptosis, phosphorylation of FADD atp serine 194 was reported to be involved in cell cycle progression[5-8]. Hua and coworkers showed that phosphorylation of FADD is

essential for growth/proliferation in T cells[7]. In human cell lines it was reported that phosphorylation of FADD caused more cells to be arrested in the G2/M phase of the cell cycle[6,9]. Cells in the G2/M phase are more radiosensitive than cells in other phases of the cell cycle[10]. The fact that phosphorylation of FADD is associated with nuclear localisation in our tumours and involved in G2/M arrest in in-vitro models[9], suggests that FADD might play an important role in sensitizing these tumours for radiation. However, whether pFADD itself triggers cells into G2/M arrest or cells at G2/M arrest show expression of pFADD has to be tested in future experiments by knocking-down or expressing FADD and pFADD mutants in HNSCC cell lines and determine directly radiotherapy response by the classical clonogenic survival assay.

Furthermore, the role of FADD in cell cycle regulation suggests that FADD is also implicated in the response to cytotoxic drugs. Allapat and coworkers reported that the ability of FADD to arrest cells in G2/M requires an intact Ser194 phosphorylation site. In addition, they showed that phosphorylation of Ser194 in FADD synergizes with the ability of Taxol to induce a G2/M cell cycle arrest[6]. Shimada and coworkers have shown that the phosphorylation status of Ser194 in FADD determines the sensitivity of cancer cells to chemotherapeutic drugs[11,12]. In a recent study, Jang and coworkers reported that the oncoprotein Polo-like kinase 1 (Plk1) phosphorylates FADD at Ser194 in response to treatment with Taxol[13]. pFADD degrades Plk1, establishing a negative feedback loop and overexpression of pFADD arrests the cell cycle. In another study, Jang and coworkers reported on a novel phosphorylation site of FADD at Ser203 in addition to the Ser194 site in response to Taxol treatment[14]. Furthermore, FADD was double phosphorylated by sequential action of Aur-A and Plk1 upon Taxol treatment, activating both caspase-dependent and caspase-independent pathways leading to cell death[14].

In advanced stage HNSCC the combined use of radiotherapy and chemotherapy has proved more efficient than radiotherapy alone[15]. Our results show a better local control in tumours overexpressing pFADD treated with radiotherapy alone. Possibly, patients with laryngeal carcinoma might further benefit from combined

chemotherapy (Taxol) and radiotherapy. To validate our findings and hypothesis we might study pFADD expression levels in an independent series of LSCC treated with radiotherapy versus a group treated with radiotherapy/chemotherapy. So far, such studies are not available.

Hypoxia Inducible Factor 1α (HIF1α), Carbonic Anhydrase IX (CA-IX)

Tumour hypoxia is frequently seen in HNSCC and is related to radioresistance and therefore worse locoregional tumour control and survival after radiotherapy[16,17]. Several techniques are available to measure tumour hypoxia, including clinical invasive measurement using needle electrodes[18,19], less invasive methods like exogenous markers (pimonidazole and EF5)[20,21], biological hypoxic tracer imaging with F-MISO or FAZA PET scan[22-24], plasma measurement of osteopontin[25] and endogenous hypoxia related tumour markers (HIF1α, HIF2a, GLUT-1, GLUT-3, CA-IX)[26,27]. The question arises which is the optimal method for measuring tumour hypoxia. It is difficult to compare different measurement techniques because correlation between these methods is not always clear and thus it is not clear what would be the golden standard. Several studies showed a poor to moderate correlation between Pimonidazol and endogenous tumour markers, although they might identify different populations of tumour cells and therefore maybe complementary[28,29]. A recent study by Mortensen and coworkers did not show a correlation between F-MISO PET and oxygen measurement by Eppendorf electrode[22]. In many studies the hypoxic measurement by Eppendorf electrodes is regarded as the gold standard. The main disadvantages of this method are its invasiveness in tumour tissues and the fact that many tumours are not accessible with the electrodes, like tumours in the glottic larynx. Since early stage glottic carcinomas are not accessible with electrodes, the use of endogenous tumour markers might be more appropriate, since these markers can be tested on the same pre-treatment biopsy samples used for histopathological classification, and do not need intravenous administration of the antibody. Studies on patients with cervical

cancer treated with radiotherapy showed a good correlation between oxygenation status measured by Eppendorf electrode and the expression of the endogenous hypoxia markers HIF1α and CA-IX[30,31]. In **chapter 5** we studied the prognostic value of three endogenous markers (HIF1α, CA-IX and GLUT-1) in a group of early stage glottic carcinomas primarily treated with radiotherapy only. We showed that high HIF1α and CA-IX expression are possibly significant prognostic factors for local recurrence in early stage glottic carcinomas. More importantly, we identified a subgroup of glottic laryngeal carcinomas with a very high local control rate after radiotherapy, i.e. the cases with low expression of both HIF1α and CA-IX.

In a recent systematic review and meta-analysis a significant improved loco-regional control, disease specific survival and overall survival in patients treated with hypoxic modification of radiotherapy was reported in unselected patients with HNSCC[32]. The author states that there is level 1A evidence in favour of adding hypoxic modification to radiotherapy in HNSCC. Possibly, the results would have been even better if hypoxic modification would have been given only in a selected group with proven tumour hypoxia. Recently, it was hypothesized that a predictive hypoxic gene profile, including more hypoxia-responsive genes, would be more robust than a single hypoxia marker[33]. Toustrup and coworkers were the first to report on a 15-gene hypoxia classifier with not only prognostic but also predictive impact[34]. This 15-gene hypoxia classifier identified patients with improved clinical outcome after hypoxic modification and radiotherapy.

Our study demonstrates that patients with early stage glottic carcinoma overexpressing HIF1α and/or CA-IX might benefit from hypoxic modification in combination with curative radiotherapy, but at the moment, hypoxic modification is not used as a standard treatment in combination with radiotherapy for patients with early stage glottis carcinoma in our institute.

Because of the possible predictive value of hypoxia for treatment modification/ stratification, pre-operative PET-scan imaging to select patients with hypoxic tumour features would be useful. In order to test whether FAZA PET is associated

with the endogenous hypoxia markers HIF1α and CA-IX, in our research group a pilot study was initiated to measure hypoxia by FAZA PET, Pimonidazole immunohistochemical staining and HIF1α /CA-IX staining in a group of 12 patients with advanced stage/recurrent laryngeal carcinomas.

The epidermal growth factor receptor (EGFR)

Overexpression of EGFR is frequently seen in a variety of malignancies, and is reported in 80% of all HNSCC's[35]. EGFR (also referred to as ErbB1) belongs to the ErbB family of tyrosine kinases which also include ErbB2 (HER-2/Neu), ErbB3 (HER 3) and ErbB4 (HER-4)[36]. Activation of EGFR signaling pathways is reported to lead to increased proliferation, metastasis, angiogenesis and decreased apoptosis in tumour cells[37,38]. Furthermore, EGFR overexpression is associated with resistance of tumour cells against chemo- and radiotherapy and thereby poor clinical outcome[39-41]. There are several possible explanations for this association. Ionizing radiation leads to autophosphorylation and activation of the tyrosine kinase domain of EGFR with activation of downstream signaling pathways[42]. The two pathways mainly responsible for resistance to chemo- and radiotherapy are the RAS/MAPK and PI3K/PTEN/AKT pathways. These pathways play a role in increased cell survival by inhibiting apoptosis (PI3K/PTEN/AKT) and increased cell proliferation (RAS/MAPK)[37]. This leads to accelerated repopulation of tumour cells after exposure to ionizing radiation[43]. In a preclinical study, Dent et al showed that irradiation could lead to activation of EGFR associated with increased proliferation and increased repopulation during radiotherapy[44]. Therefore, activation of EGFR induced by irradiation may lead to increased radioresistance in HNSCC. More recently, Rodemann et al. reported that EGFR activation plays a role in DNA-double strand break repair after radiotherapy[37].

There are several ways to reduce the negative effect of EGFR overexpression/ activation in patients treated with radiotherapy. To reduce the increased repopulation as a result of autophosphorylation of EGFR, patients can be treated

with an accelerated radiotherapy schedule. Two studies showed that local control in patients overexpressing EGFR was better when treated with accelerated RTH compared to conventional RTH[45,46] . Another strategy to reduce the negative effects of EGFR overexpression/activation is the inhibition of EGFR activity during radiotherapy. Therefore, two strategies have been developed. The first targets the intracellular domain of the receptor by blocking the ATP binding site in the tyrosine kinase domain of EGFR by the use of tyrosine kinase inhibitors gefitinib and erlotinib[47,48]. The second is the use of a monoclonal antibody against EGFR, (cetuximab), which targets the extracellular domain of the EGFR[43,49]. Of these anti-EGFR agents, cetuximab is studied the most in HNSCC. The binding of cetuximab to the EGFR leads to internalization and degradation of the antibody-receptor complex, consequently downregulating EGFR expression[43,49]. In a large, multinational, randomized study Bonner and coworkers showed that in a group of locoregional advanced stage HNSCC the use of cetuximab plus radiotherapy resulted in better locoregional control and survival compared to patients treated with radiotherapy alone[50,51].

Despite the intitial excitement after the start of the use of EGFR inhibitors, the clinical benefit has been somewhat disappointing. Different pre-clinical studies have suggested multiple mechanisms which may lead to EGFR blockage resistance[52,53]. There are differences in the mechanisms of resistance in different tumour types. To overcome EGFR blockage resistance the use of combined therapy targeting multiple ErbB receptors has been studied recently in different tumour types[54-56]. Whether this will lead to better local control and survival rates has to be seen. In **chapter 6** we studied whether EGFR expression was associated with clinical outcome in patients with early stage supraglottic and glottic carcinoma. We described an association between high EGFR expression and positive lymph node status. Ninety percent of all patients with pre-treatment positive lymph node status showed high EGFR expression. In good agreement with our observation, the activation of EGFR was reported to lead to increased metastasis and proliferation[37,57]. Furthermore, we showed that high EGFR expression was

associated with LSCC located in the supraglottis. In the T2 supraglottic tumour group a trend towards worse local control and overall survival in the group overexpressing EGFR was observed. Possibly the lymph-node-positive supraglottic carcinomas will benefit from accelerated RT combined with EGFR-targeted therapy. So far, no studies have been published which compare concomitant cetuximab and radiation therapy and/or in combination with EGFR levels in early stage laryngeal carcinoma. This might be subject of a future study.

CONCLUSION

This thesis shows new insights in the molecular mechanisms of response to radiotherapy and clinical outcome in mainly early stage laryngeal carcinomas. With the development of predictive molecular profiles, it might be possible to choose the optimal treatment strategy for the individual patient. Our results may contribute to the selection of patients who might benefit from adding chemotherapy, EGFR blocking agents or hypoxic modification to the current standard (accelerated) radiotherapy. However, it is difficult to compare our results with the current literature because of differences in methodology. Therefore, to be implemented in the daily clinical practice our results have to be validated in an independent dataset.

REFERENCES

1. Gibcus JH, Menkema L, Mastik MF, et al. Amplicon mapping and expression profiling identify the fas-associated death domain gene as a new driver in the 11q13.3 amplicon in laryngeal/ pharyngeal cancer. *Clin Cancer Res*. 2007;13(1078-0432; 21):6257-6266.
2. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev*. 1993;7(0890-9369; 5):812-821.
3. Morshed K, Skomra D, Korobowicz E, Szymanski M, Polz-Dacewicz M, Golabek W. An immunohistochemical study of cyclin D1 protein expression in laryngeal squamous cell carcinoma. *Acta Otolaryngol*. 2007;127(0001-6489; 7):760-769.
4. van Rossum AG, Moolenaar WH, Schuuring E. Cortactin affects cell migration by regulating intercellular adhesion and cell spreading. *Exp Cell Res*. 2006;312(0014-4827; 0014-4827; 9): 1658-1670.
5. Alappat EC, Feig C, Boyerinas B, et al. Phosphorylation of FADD at serine 194 by CKIalpha regulates its nonapoptotic activities. *Mol Cell*. 2005;19(1097-2765; 3):321-332.
6. Alappat EC, Volkland J, Peter ME. Cell cycle effects by C-FADD depend on its C-terminal phosphorylation site. *J Biol Chem*. 2003;278(0021-9258; 43):41585-41588.
7. Hua ZC, Sohn SJ, Kang C, Cado D, Winoto A. A function of fas-associated death domain protein in cell cycle progression localized to a single amino acid at its C-terminal region. *Immunity*. 2003;18(1074-7613; 4):513-521.
8. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of fas and initiates apoptosis. *Cell*. 1995;81(4):505-512.
9. Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Konishi N. Phosphorylation status of fas-associated death domain-containing protein (FADD) is associated with prostate cancer progression. *J Pathol*. 2005;206(0022-3417; 4):423-432.
10. Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys*. 2004;59(0360-3016; 4):928-942.
11. Shimada K, Nakamura M, Ishida E, Kishi M, Yonehara S, Konishi N. Phosphorylation of fas-associated death domain contributes to enhancement of etoposide-induced apoptosis in prostate cancer cells. *Jpn J Cancer Res*. 2002;93(0910-5050; 0910-5050; 10):1164-1174.

12. Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Kishi M, Konishi N. Phosphorylation of FADD is critical for sensitivity to anticancer drug-induced apoptosis. *Carcinogenesis*. 2004;25(7):1089-1097.

13. Jang MS, Lee SJ, Kim CJ, Lee CW, Kim E. Phosphorylation by polo-like kinase 1 induces the tumor-suppressing activity of FADD. *Oncogene*. 2011;30(4):471-481.

14. Jang MS, Lee SJ, Kang NS, Kim E. Cooperative phosphorylation of FADD by aur-A and Plk1 in response to taxol triggers both apoptotic and necrotic cell death. *Cancer Res*. 2011;71(23): 7207-7215.

15. Bernier J, Domenge C, Ozsahin M, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. *N Engl J Med*. 2004;350(19): 1945-1952.

16. Hoogsteen IJ, Marres HA, van der Kogel AJ, Kaanders JH. The hypoxic tumour microenvironment, patient selection and hypoxia-modifying treatments. *Clin Oncol (R Coll Radiol)*. 2007(0936-6555).

17. Vaupel P, Mayer A. Hypoxia in cancer: Significance and impact on clinical outcome. *Cancer Metastasis Rev*. 2007(0167-7659).

18. Nordsmark M, Bentzen SM, Rudat V, et al. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. an international multi-center study. *Radiother Oncol*. 2005;77(1):18-24.

19. Horsman MR, Khalil AA, Siemann DW, et al. Relationship between radiobiological hypoxia in tumors and electrode measurements of tumor oxygenation. *Int J Radiat Oncol Biol Phys*. 1994;29(3):439-442.

20. Yaromina A, Thames H, Zhou X, et al. Radiobiological hypoxia, histological parameters of tumour microenvironment and local tumour control after fractionated irradiation. *Radiother Oncol*. 2010;96(1):116-122.

21. Kaanders JH, Wijffels KI, Marres HA, et al. Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. *Cancer Res*. 2002;62(23):7066-7074.

22. Mortensen LS, Buus S, Nordsmark M, et al. Identifying hypoxia in human tumors: A correlation study between 18F-FMISO PET and the eppendorf oxygen-sensitive electrode. *Acta Oncol*. 2010;49(7):934-940.

23. Rajendran JG, Schwartz DL, O'Sullivan J, et al. Tumor hypoxia imaging with [F-18] fluoromisonidazole positron emission tomography in head and neck cancer. *Clin Cancer Res*. 2006;12(1078-0432; 18):5435-5441.

24. Grosu AL, Souvatzoglou M, Roper B, et al. Hypoxia imaging with FAZA-PET and theoretical considerations with regard to dose painting for individualization of radiotherapy in patients with head and neck cancer. *Int J Radiat Oncol Biol Phys*. 2007;69(0360-3016; 0360-3016; 2): 541-551.

25. Overgaard J, Eriksen JG, Nordsmark M, Alsner J, Horsman MR. Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: Results from the DAHANCA 5 randomised double-blind placebo-controlled trial. *Lancet Oncol*. 2005;6(1470-2045; 10):757-764.

26. Hoogsteen IJ, Marres HA, Bussink J, van der Kogel AJ, Kaanders JH. Tumor microenvironment in head and neck squamous cell carcinomas: Predictive value and clinical relevance of hypoxic markers. A review. *Head Neck*. 2007;29(1043-3074; 6):591-604.

27. Bussink J, Kaanders JH, van der Kogel AJ. Tumor hypoxia at the micro-regional level: Clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. *Radiother Oncol*. 2003;67(0167-8140; 1):3-15.

28. Hoogsteen IJ, Lok J, Marres HA, et al. Hypoxia in larynx carcinomas assessed by pimonidazole binding and the value of CA-IX and vascularity as surrogate markers of hypoxia. *Eur J Cancer*. 2009;45(16):2906-2914.

29. Jankovic B, Aquino-Parsons C, Raleigh JA, et al. Comparison between pimonidazole binding, oxygen electrode measurements, and expression of endogenous hypoxia markers in cancer of the uterine cervix. *Cytometry B Clin Cytom*. 2006;70(2):45-55.

30. Haugland HK, Vukovic V, Pintilie M, et al. Expression of hypoxia-inducible factor-1alpha in cervical carcinomas: Correlation with tumor oxygenation. *Int J Radiat Oncol Biol Phys*. 2002;53(0360-3016; 4):854-861.

31. Loncaster JA, Harris AL, Davidson SE, et al. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: Correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res*. 2001;61(0008-5472; 17): 6394-6399.

32. Overgaard J. Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck--a systematic review and meta-analysis. *Radiother Oncol*. 2011;100(1):22-32.

33. Toustrup K, Sorensen BS, Alsner J, Overgaard J. Hypoxia gene expression signatures as prognostic and predictive markers in head and neck radiotherapy. *Semin Radiat Oncol*. 2012;22(2):119-127.

34. Toustrup K, Sorensen BS, Lassen P, et al. Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. *Radiother Oncol*. 2012;102(1):122-129.

35. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res*. 1993;53(0008-5472; 15):3579-3584.

36. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001;2(2):127-137.

37. Rodemann HP, Dittmann K, Toulany M. Radiation-induced EGFR-signaling and control of DNA-damage repair. *Int J Radiat Biol*. 2007;83(11-12):781-791.

38. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*. 2005;9(1):59-71.

39. Krause M, Prager J, Zhou X, et al. EGFR-TK inhibition before radiotherapy reduces tumour volume but does not improve local control: Differential response of cancer stem cells and nontumourigenic cells? *Radiother Oncol*. 2007;83(3):316-325.

40. Krause M, Zips D, Thames HD, Kummermehr J, Baumann M. Preclinical evaluation of molecular-targeted anticancer agents for radiotherapy. *Radiother Oncol*. 2006;80(2):112-122.

41. Normanno N, De Luca A, Bianco C, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene*. 2006;366(1):2-16.

42. Park CM, Park MJ, Kwak HJ, et al. Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways. *Cancer Res*. 2006;66(17): 8511-8519.

43. Zimmermann M, Zouhair A, Azria D, Ozsahin M. The epidermal growth factor receptor (EGFR) in head and neck cancer: Its role and treatment implications. *Radiat Oncol*. 2006;1:11.

44. Dent P, Reardon DB, Park JS, et al. Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. *Mol Biol Cell*. 1999;10(8):2493-2506.

45. Eriksen JG, Steiniche T, Overgaard J. The role of epidermal growth factor receptor and E-cadherin for the outcome of reduction in the overall treatment time of radiotherapy of supraglottic larynx squamous cell carcinoma. *Acta Oncol*. 2005;44(1):50-58.

46. Bentzen SM, Atasoy BM, Daley FM, et al. Epidermal growth factor receptor expression in pretreatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. *J Clin Oncol*. 2005;23(0732-183; 24):5560-5567.

47. Cohen EE, Kane MA, List MA, et al. Phase II trial of gefitinib 250 mg daily in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. *Clin Cancer Res*. 2005;11(23):8418-8424.

48. Soulieres D, Senzer NN, Vokes EE, Hidalgo M, Agarwala SS, Siu LL. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. *J Clin Oncol*. 2004;22(1): 77-85.

49. Machiels JP, Subramanian S, Ruzsa A, et al. Zalutumumab plus best supportive care versus best supportive care alone in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck after failure of platinum-based chemotherapy: An open-label, randomised phase 3 trial. *Lancet Oncol*. 2011;12(4):333-343.

50. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol*. 2010;11(1):21-28.

51. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006;354(1533-4406; 6):567-578.

52. Rabinowits G, Haddad RI. Overcoming resistance to EGFR inhibitor in head and neck cancer: A review of the literature. *Oral Oncol*. 2012.

53. Quesnelle KM, Wheeler SE, Ratay MK, Grandis JR. Preclinical modeling of EGFR inhibitor resistance in head and neck cancer. *Cancer Biol Ther*. 2012;13(10):935-945.

54. Cohen EE, Davis DW, Karrison TG, et al. Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: A phase I/II study. *Lancet Oncol*. 2009;10(3):247-257.

55. Nakagawa T, Takeuchi S, Yamada T, et al. Combined therapy with mutant-selective EGFR inhibitor and met kinase inhibitor for overcoming erlotinib resistance in EGFR mutant lung cancer. *Mol Cancer Ther*. 2012.

56. Wang M, Zhao J, Zhang LM, et al. Combined erlotinib and cetuximab overcome the acquired resistance to epidermal growth factor receptors tyrosine kinase inhibitor in non-small-cell lung cancer. *J Cancer Res Clin Oncol*. 2012.

57. Ang KK, Berkey BA, Tu X, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res*. 2002;62(0008-5472; 24):7350-7356.

Chapter 8

Summary

SUMMARY

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer in the world with an incidence of over 600.000 cases per year and a mortality of 350.000 per year Twenty percent of all head and neck tumours originate in the larynx, of which the majority is located in the glottic region. Most patients with glottic carcinoma present with early stage disease (T1/T2). Most early stage (T1/T2) laryngeal carcinomas are treated with conservative (laser) surgery or (accelerated) radiotherapy. For more advanced tumours (T3/T4), surgery and/or chemoradiotherapy is the treatment of choice. Recently, for advanced stage tumours a combination of an EGFR blocking agent (Cetuximab, Gefitinib) and radiotherapy has been used. Until now, the choice of treatment is mainly based on well known clinicopathological factors, such as tumour location and tumour stage. Besides the TNM classification few prognostic factors are available to predict clinical outcome in terms of locoregional control, overall survival or disease specific survival. Cell biological tumour markers associated with response to treatment or prognosis might be useful to predict clinical outcome before treatment, and thereby optimize and personalize the treatment for individual patients. In this thesis, cell biological tumour markers involved in locoregional control, overall survival, disease specific survival and lymph node status in patients with laryngeal squamous cell carcinoma treated with radiotherapy were investigated. Furthermore, we evaluated different treatment modalities used in our institute in the past.

Clinical outcome of endoscopic CO_2 laser surgery and radiotherapy in early stage glottic laryngeal carcinoma is difficult to compare because of differences in treatment selection and patient groups. Therefore in **chapter 2**, we compared local control, overall survival and laryngeal preservation in a homogenous group of patients with T1a glottic carcinoma with normal/diminished mucosal wave treated either with CO_2 laser surgery or radiotherapy. Retrospective survival analysis was performed on 100 patients with T1a glottic carcinoma treated with CO_2 laser surgery (n=49) or radiotherapy (n=51) diagnosed at the University Medical Center

Groningen between 1990-2004. Kaplan Meier analysis was performed to measure differences in local control, overall survival and laryngeal preservation between the radiotherapy and CO_2 laser group. No significant differences in local control and overall survival were found between the CO_2 laser surgery and radiotherapy group. Ultimate 5-year laryngeal preservation was significantly better in the CO_2 laser surgery group (95% vs. 77%, p=0.043). These results show that in a very well defined subset consisting only of T1a glottic laryngeal carcinoma with normal or diminished mucosal wave CO_2 laser surgery is preferred over radiotherapy as the primary treatment because of the better laryngeal preservation rate. Besides, salvage radiotherapy can be used after primary CO_2 laser therapy for local recurrences, and the ultimate salvage total laryngectomy can be reserved for recurrences after salvage radiotherapy treatment.

In **chapter 3** we evaluated whether the clinical introduction of accelerated radiation therapy (ART) in T2-T4 laryngeal carcinoma improved outcome in our institute. Until 2000, at the department of Radiation Oncology of the University Medical Center Groningen (UMCG), patients with T2-T4 laryngeal carcinoma were treated with conventional fractionated radiotherapy (CF) consisting of 2 Gy per fraction to a total dose of 66 Gy to 70 Gy, 5 times per week, over a period of 7 weeks. After the publication of a number of randomized controlled trials, accelerated radiotherapy was increasingly used as the new standard in case chemoradiation was considered not feasible. Retrospectively, 181 patients with T2b-T4 glottic or T2-T4 supraglottic laryngeal carcinoma primarily treated with CF, or ART who were treated at the UMCG were included. Univariate Kaplan meier analysis and multivariate analysis using the Cox proportional hazards model was performed to compare the two fractionation schedules in a single centre setting with regard to local control, overall survival and disease specific survival. In the multivariate analysis, LC was significantly better in patients treated with ART than those treated with CF (HR 1.76, 95% CI 1.01-3.05). Other independent prognostic factors associated with better local control were N0-status (HR 2.13, 95% CI 1.14-3.98) and female sex (HR 2.69, 95% CI 1.25-5.76). Improved local control

did not translate into a significant improvement of the overall survival or disease specific survival. Our results confirmed that local control in T2-T4 laryngeal carcinoma improved after changing the institutional policy from CF to ART.

In **chapter 4** protein expression of the Fas-associated death domain (FADD) and its Ser194-phosphorylated isoform (pFADD) was studied and their prognostic value on local control was determined. For this purpose, immunohistochemical staining for FADD, pFADD, cortactin and cyclin D1 was performed on pretreatment biopsies of ninety-two patients with T1-T2 glottic squamous cell carcinoma primarily treated with radiotherapy between 1996 and 2005. Cox regression analysis was performed to correlate expression levels with local control.

High levels of pFADD were associated with a significant better local control (HR 2.40, 95% CI 1.04-5.55, p=0.040). FADD overexpression showed a trend towards better local control (HR 3.656; 95% CI 0.853-15.663, p=0.081). Cortactin and cyclin D1 were not associated with local control. Multivariate Cox regression analysis revealed that high pFADD was the best predictor for local control after radiotherapy. This shows that expression of phosphorylated FADD is a new prognostic biomarker for a better local control after radiotherapy in patients with early stage glottic carcinomas.

In **chapter 5** the prognostic value of three endogenous hypoxia markers (HIF1α, CA-IX and GLUT-1) on clinical outcome was studied and a predictive hypoxic profile to choose the optimal treatment in early stage laryngeal carcinoma was examined. For this purpose, immunohistochemistry for HIF1α, CA-IX and GLUT-1 was performed on formalin-fixed paraffin-embedded pre-treatment tissue samples of 91 glottic squamous cell carcinomas. The patient group consisted only of early stage (T1/T2) glottic carcinomas, and all patients were treated with radiotherapy only. Relative tumour staining was scored on the tissue samples. Kaplan-Meier survival analysis and Cox regression analysis for the variables HIF1α, CA-IX, GLUT-1 was performed to correlate expression levels with local control and overall survival.

HIF1α overexpression significantly correlated with worse local control (HR 3.05, 95% CI 1.18-7.86, p=0.021) and overall survival (HR 2.92, 95% CI 1.22-6.99, p=0.016). CA-IX overexpression was significantly correlated with worse local control (HR 2.93, 95% CI 1.18-7.26, p=0.020). GLUT-1 overexpression did not show any correlation with clinical outcome parameters.Tumours showing a non-hypoxic profile (defined as low HIF1α and low CA-IX) were significantly correlated to a better local control (HR 6.32, 95% CI 1.47-27.15, p=0.013).

In conclusion, the results in this chapter indicate that CA-IX and HIF1α are possibly significant prognostic markers for local control in patients with T1/T2 glottic carcinoma treated with radiotherapy only. In this specific subset of patients, the addition of hypoxic modification should be considered.

Finally, in **chapter 6** we examined the prognostic effect of the epidermal growth factor receptor (EGFR) expression on local control, survival and lymph node metastasis. Therefore, immunohistochemical staining for EGFR was performed on pre-treatment biopsies of 139 patients with T1/T2 laryngeal squamous cell carcinoma primarily treated with radiotherapy between 1996 and 2005. Logistic regression, Kaplan Meier analysis and Cox regression analysis was performed to correlate expression levels with local control, overall survival, disease specific survival and lymph node status. EGFR overexpression was significantly associated with positive N status and supraglottic tumour location (OR 8.71, 95% CI 1.12-67.93, p=0.039 and OR 14.56, 95% CI 4.22-50.28, p=0.000 respectively). Kaplan Meier survival analysis and Cox regression analysis did not show a significant relation between EGFR expression and local control (HR 1.19, 95% CI 0.58-2.42, p=0.64), overall survival (HR 0.62, 95% CI 0.31-1.22, p=0.16) and disease specific survival respectively (HR 1.19, 95% 0.33-4.34, p=0.79). These results showed a significant correlation between EGFR overexpression and supraglottic tumour location and lymph node positivity. These groups might benefit from adding an EGFR blocking agent to the accelerated RT. No relation between

EGFR overexpression and local control, overall survival and disease specific survival was seen.

In this thesis we studied different biological tumour markers in laryngeal carcinoma treated with radiotherapy, and correlated them with clinical outcome parameters as local control, overall survival, disease specific survival and lymph node status. **Chapter 7** provides a general discussion in which the results described in chapters 4, 5 and 6 are summarized and are compared with the current literature. Furthermore, the cell biological background of the tested tumour markers is described. Finally, possible clinical implications of our results are discussed.

Chapter 9

Nederlandse samenvatting

SAMENVATTING

Kanker in het hoofd/hals gebied is de vijfde meest voorkomende vorm van kanker in de wereld. Jaarlijks worden 600.000 nieuwe patiënten gediagnosticeerd en sterven er 350.000 patiënten aan deze vorm van kanker. Twintig procent van alle hoofd- hals tumoren zijn gelokaliseerd in de larynx, waarvan ongeveer 66% op de ware stembanden (glottis), 31% op de valse stembanden (supraglottis) en 2% onder de stembanden (subglottis). Het merendeel van de patiënten met een glottistumor presenteert zich in een vroeg stadium (T1/T2 tumor). De primaire klacht is dikwijls een persisterende heesheid. De meeste kleine glottistumoren (T1/T2) worden door middel van chirurgie of (geaccelereerde) radiotherapie behandeld. Een geselecteerde groep T1a glottistumoren met een intacte of afgenomen randgolf van de ware stemplooi wordt behandeld door middel van CO_2 laserchirurgie. Voor de meer uitgebreide tumoren (T3/T4) is chirurgie en/of chemoradiotherapie de eerste keus behandeling. Recent is ook een combinatie van een EGFR blokker (Cetuximab, Gefitinib) met radiotherapie beschreven voor grotere tumoren. Tot nu toe hangt de keuze voor de behandeling af van bekende klinische en pathologische factoren, zoals tumor lokalisatie en stadium. Om de behandeling voor individuele patiënten te optimaliseren, is het nodig om te kunnen voorspellen of een tumor reageert op een bepaald soort behandeling. Momenteel zijn er echter naast de TNM classificatie weinig prognostische factoren die de locoregionale controle, totale overleving en ziektespecifieke overleving kunnen voorspellen. Celbiologische tumormarkers zijn mogelijk bruikbaar als voorspellers van het effect van radiotherapie.

In dit proefschrift is de relatie onderzocht tussen enkele celbiologische tumormarkers en de locoregionale controle, totale en ziektespecifieke overleving in een groep patiënten met larynxcarcinoom die primair zijn behandeld met radiotherapie. Daarnaast werden verschillende behandelingen geëvalueerd bij patiënten die in het verleden in onze kliniek werden behandeld voor een larynxcarcinoom.

Het is lastig om het verschil in klinische uitkomst te vergelijken tussen CO_2 laserchirurgie en radiotherapie bij patiënten met een vroeg stadium larynxcarcinoom. De reden hiervoor is dat er in de literatuur grote verschillen worden beschreven in patiëntenpopulaties en criteria om een bepaalde behandeling te kiezen. Daarom hebben we in **hoofdstuk 2** het verschil in lokale controle, overleving en larynxpreservatie bekeken in een groep patiënten met een T1a glottisch larynxcarcinoom die behandeld zijn met CO_2 laserchirurgie of radiotherapie. Er werd een retrospectieve analyse verricht van 100 patiënten met een T1a glottisch larynxcarcinoom met een normale of afgenomen randgolf gemeten met videolaryngostroboscopie. Van die 100 patiënten werden 49 behandeld met CO_2 laserchirurgie en 51 met primaire radiotherapie. Alle patiënten werden gediagnosticeerd tussen 1990 en 2004 in het Universitair Medisch Centrum Groningen (UMCG). Kaplan Meier analyse werd verricht om verschillen in lokale controle, overleving en larynxpreservatie te meten tussen de CO_2 lasergroep en radiotherapiegroep. De 5-jaars larynxpreservatie was significant beter in de groep patiënten behandeld met CO_2 laser (95% vs. 77%, p=0.043). Er werden geen verschillen gezien in lokale controle en overleving tussen beide groepen. Dit resultaat laat zien dat in een goed gedefinieerde groep patiënten met een T1a glottisch larynxcarcinoom met een normale of verminderde randgolf CO_2 laserchirurgie de voorkeur geniet boven radiotherapie. De reden hiervoor is dat radiotherapie als tweede behandeling kan worden gebruikt indien er een recidief optreedt na initiële CO_2 laserbehandeling. Een operatieve verwijdering van het strottehoofd (totale laryngectomie) kan dan worden gereserveerd voor recidieven na de aanvullende behandeling met radiotherapie.

In **hoofdstuk 3** is onderzocht of de klinische introductie van geaccelereerde radiotherapie tot een betere klinische uitkomst leidt bij patiënten met een T2-T4 larynxcarcinoom in onze kliniek. Tot 2000 werd patiënten met een T2-T4 larynxcarcinoom op de afdeling radiotherapie behandeld door middel van een conventioneel gefractioneerd schema bestaande uit 2 Gy per fractie, 5 keer per week, gedurende een periode van 7 weken, tot een totale dosis van 66 tot 70 Gy.

Na de publicatie van een aantal gerandomiseerd gecontroleerde onderzoeken, wordt geaccelereerde radiotherapie steeds meer gebruikt als de nieuwe standaard. Hierbij wordt 6 keer per week een fractie van 2 Gy gegeven, tot een totale dosis van 66 tot 70 Gy, waarbij de totale behandelduur verkort wordt.

Retrospectief werden 181 patiënten met een T2b-T4 glottisch larynxcarcinoom of een T2-T4 supraglottisch larynxcarcinoom geïncludeerd in het onderzoek. Alle patiënten werden behandeld met conventionele radiotherapie (voor 2000), of geaccelereerde radiotherapie. Als uitkomstmaten vergeleken we in beide groepen de lokale controle, totale overleving en ziektespecifieke overleving. Er was een significant betere lokale controle in de groep patiënten behandeld met geaccelereerde radiotherapie (HR 1.76, 95% CI 1.01 tot 3.05). Andere onafhankelijke prognostische factoren die een betere lokale controle lieten zien waren negatieve lymfeklierstatus (HR 2.13, 95% CI 1.14-3.98) en vrouwelijk geslacht (HR 2.69, 95% CI 1.25 tot 5.76). De betere lokale controle in de geaccelereerde groep patiënten leidde niet tot een betere overleving of ziektespecifieke overleving. Deze resultaten bevestigen dat het veranderen van het beleid van conventionele radiotherapie naar geaccelereerde radiotherapie in ons instituut heeft geleid tot een betere lokale controle.

In **hoofdstuk 4** werd de prognostische waarde van de eiwitexpressie van het Fas-associated death domain (FADD) en zijn gefosforyleerde isoform (pFADD) op lokale controle onderzocht. Hiervoor werd eiwitexpressie van FADD, pFADD, Cortactin en Cycline D1 voorafgaand aan de behandeling bepaald door middel van immunohistochemie op tumorbiopten van 92 patiënten met T1-T2 glottisch larynxcarcinoom. Alle patiënten werden behandeld met radiotherapie tussen 1996-2005. Met Cox regressie analyse werd de eiwitexpressie gerelateerd aan lokale controle. Een hoog niveau van pFADD was geassocieerd met een significant betere lokale controle (HR 2.40, 95% CI 1.04-5.55, $p = 0.040$). Hoge FADD expressie liet een trend zien naar betere lokale controle (HR 3.66; 95% CI 0.85-15.66, $p = 0.081$). Cortactin en Cycline D1 expressie lieten geen significante relatie zien met lokale controle. Multivariate Cox regressie analyse liet zien dat een

hoge pFADD waarde de beste voorspeller voor lokale controle na radiotherapie was. Concluderend lijkt een hoge pFADD waarde een nieuwe prognostische biomarker te zijn voor een betere lokale controle na radiotherapie bij patiënten met een T1-T2 glottisch larynxcarcinoom.

Uit eerder onderzoek is gebleken dat een relatief zuurstofgebrek in een tumor (hypoxie) een negatieve invloed kan hebben op lokale controle en overleving. In **hoofdstuk 5** werd de prognostische waarde van drie endogene hypoxiemarkers (HIF1α, CA-IX en GLUT-1) op de klinische uitkomst onderzocht. Uit een combinatie van deze markers werd een hypoxisch profiel opgesteld om te kunnen voorspellen of patiënten een goede genezingskans hadden na radiotherapie. Immunohistochemie werd toegepast voor HIF1α, CA-IX en GLUT-1 op onbehandeld tumorweefsel van 91 patiënten met een T1-T2 glottisch larynxcarcinoom. Alle patiënten werden met alleen radiotherapie. Kaplan Meier analyse en Cox regressie analyse voor de variabelen HIF1α, CA-IX en GLUT-1 werd uitgevoerd waarbij de expressie werd geassocieerd met de lokale controle en totale overleving. HIF1α overexpressie was significant geassocieerd met een slechtere lokale controle (HR 3.05, 95% CI 1.18-7.86, p = 0.021) en slechtere overleving (HR 2.92, 95% CI 1.22-6.99, p = 0.016). CA-IX overexpressie was significant geassocieerd met een slechtere lokale controle (HR 2.93, 95% CI 1.18-7.26, p = 0.020). GLUT-1 overexpressie liet geen verband zien met de klinische uitkomst parameters. Tumoren met een niet-hypoxisch profiel (gedefinieerd als lage HIF1α en lage CA-IX) bleken een significant betere lokale controle (HR 6.32, 95% CI 1.47-27.15, p = 0.013) te hebben. De resultaten in dit hoofdstuk laten zien dat CA-IX en HIF1α mogelijk belangrijke prognostische markers zijn voor lokale controle bij patiënten met een T1/T2 glottisch carcinoom behandeld met radiotherapie. In deze specifieke selectie van patiënten kan hypoxische tumormodificatie worden overwogen.

In **hoofdstuk 6** werd de rol van de epidermal growth factor receptor (EGFR) bepaald in relatie tot lokale controle, overleving en lymfekliermetastasen. Eiwitexpressie werd bepaald door middel van immunohistochemie op

onbehandelde tumorbiopten van 139 patiënten met T1/T2 larynxcarcinoom behandeld met radiotherapie tussen 1996 en 2005. Logistische regressie, Kaplan Meier analyse en Cox regressie analyse werden uitgevoerd om eiwitexpressie te correleren met de lokale controle, algehele overleving, ziekte specifieke overleving en lymfeklier status. EGFR overexpressie bleek gecorreleerd met een positieve lymfeklierstatus en supraglottische tumorlokatie (OR 8.71, 95% CI 1.12-67.93, p = 0.039 en OR 14.56, 95% CI 4.22-50.28, p = 0,000 respectievelijk). Kaplan Meier analyse en Cox regressie analyse toonden geen significante relatie tussen de EGFR expressie en lokale controle (HR 1.19, 95% CI 0.58-2.42, p = 0.64), overleving (HR 0.62, 95% CI 0.31-1.22, p = 0.16) en ziekte specifieke overleving (HR 1.19, 95% 0.33-4.34, p = 0.79). Concluderend hebben we aangetoond dat er een significante relatie bestaat tussen EGFR overexpressie en positieve lymfeklierstatus en supraglottische tumorlokatie. Deze patiënten kunnen mogelijk baat hebben bij het toevoegen van anti-EGFR therapie aan de standaard geaccelereerde radiotherapie.

Tot slot volgt in **hoofdstuk 7** een algemene discussie waarin de resultaten van de in dit proefschrift besproken mogelijke prognostische tumormarkers worden bediscussieerd. De celbiologische achtergrond van de geteste tumormarkers wordt beschreven en onze resultaten worden besproken en vergeleken met de huidige literatuur. Verder worden de mogelijke klinische implicaties van de resultaten van dit proefschrift besproken.

Dankwoord

DANKWOORD

Dit proefschrift zou niet tot stand zijn gekomen zonder de hulp van meerdere personen. Graag wil ik iedereen bedanken die een bijdrage heeft geleverd. Een aantal personen wil ik in het bijzonder noemen.

Prof. dr. E.M.D. Schuuring. Beste Ed, boss, jij was de motor achter het biologische gedeelte van dit proefschrift. Ik heb behoorlijk aan jou en je manier van werken moeten wennen toen ik eind 2005 op de afdeling Pathologie begon als net afgestudeerd arts. Ik kwam in een voor mij onbekende wereld van TMA's, micro-RNA's, celtransfecties en andere zaken waar ik nog nooit van had gehoord. Iedere vraag die ik stelde werd beantwoord met een wedervraag, met als doel me zelf goed en kritisch na te laten denken over de stof die ik aan het lezen was. Ik zie me zelf nog zitten in de kerstvakantie, met "the basic science of oncology", om mezelf in te lezen in de celbiologische achtergrond van tumoren. Gelukkig went alles en kunnen we inmiddels al weer jaren goed met elkaar opschieten. Zeer prettig dat ik altijd langs kon komen om even te "bomen" over een artikel.
Voor mij ben jij zoals een wetenschapper hoort te zijn. Niet snel scoren. Altijd kritisch, altijd bezig met extra proeven te verzinnen welke de resultaten nog verder kunnen onderbouwen. Dank je wel voor de goede en prettige begeleiding al deze jaren. Dat had deze dokter wel nodig.

Prof. dr. B.F.A.M. van der Laan, Beste Bernard. Mijn promotor en tevens opleider. Jij hebt me 7 jaar geleden de kans te geven om te promoveren en de opleiding tot KNO-arts te volgen. Dank je wel voor je goede begeleiding, enthousiasme en interesse in zowel dit onderzoek, als in de opleiding en op persoonlijk vlak.

Dr. J.E. van der Wal, beste Jacqueline. Met name in de beginfase van het onderzoek heb ik veel tijd op jouw kamer achter de microscoop doorgebracht. Van alle patiënten die mogelijk geschikt waren voor dit proefschrif hebben we de coupes

bekeken en het tumorpercentage bepaald. Ondanks dat je redelijk snel na de start van mijn onderzoek elders werkzaam was, kwam je toch wekelijks terug voor overleg, en zorgde je ervoor dat je actief betrokken was bij het onderzoek. Dank voor je hulp en fijne samenwerking.

De beoordelingscommissie, Prof. dr. J.A. Langendijk, Prof. dr. P.M. Kluin en Prof. dr. J.L.N. Roodenburg, wil ik bedanken voor de bereidheid het manuscript deskundig te beoordelen.

Prof. dr. J.A. Langendijk, beste Hans, hartelijk dank voor je kritische beoordeling van enkele manuscripten.

Prof. dr. G.H. de Bock, beste Truuske, dank je wel voor de begeleiding in het statistische deel van het onderzoek.

Lorian Slagter-Menkema. Beste Lorian, jij bent onmisbaar geweest voor de praktische uitvoering van het geheel. Veel dank voor het snijden van de coupes, uitvoeren van de kleuringen, en alle ondersteuning op het O&O en DNA lab. Vele uren hebben we samen achter de microscoop gezeten om de kleuringen te scoren. Dank je wel voor de prettige samenwerking.

Mirjam Mastik. Beste Mirjam, hartelijk dank voor alle hulp met de kleuringen en scoren van de coupes.

Wouter Pattje. Beste Wouter, mijn kamergenoot bij de pathologie. We zijn ongeveer tegelijk begonnen met ons promotieonderzoek. Ik bij de KNO en jij bij de radiotherapie. Aangezien we zeer verwante onderwerpen hebben voor ons promotieonderzoek, hebben we zeer veel met elkaar te maken gehad. Wij hebben intensief samengewerkt bij het kweken van cellen, scoren van coupes, het bestralen van de cellen en de MTT assays. Daarnaast zijn we meerdere malen op cursus en

congres geweest, waarbij we ook bij het sociale programma een goed team bleken. Dank je wel voor de samenwerking en gezelligheid op de kamer. Succes nog met het afronden van jouw promotie.

Dr. Johan Gibcus, beste Johan, in de beginfase van mijn onderzoek (en ook later) heb ik zeer veel gehad aan jouw adviezen. Ik kon altijd bij jou terecht voor diepgaande uitleg te over de microbiologische aspecten van tumoren. Voor diepgaande analyse over de afgelopen speelronde in de eredivisie, kanshebbers in de Tour de France of Olympische spelen kon je dan weer bij mij terecht. Een deel van dit proefschrift is een vervolg op jouw pionierswerk op het 11q13 amplicon. Naast het werk hebben we mooie avonden aan de bar beleefd. Jij bent tegenwoordig hardcore onderzoeker in Boston. Ik wens je alle succes en goede resultaten toe!

Marije Bouman. Beste Marije, dank je wel voor je hulp met Acces en het bouwen van mijn database.

Tom van der Laan. Beste Tom, hartelijk dank voor je goede hulp bij de layout van het proefschrift. Ik zal voortaan de spatie links laten liggen.

Ik wil graag alle mensen van de afdeling pathologie, DNA en O&O lab bedanken: Freke, Mirjam Boelens, Sicco, Geert, Tjasso, Klaas, Jane, Rikst Nynke en alle anderen waarmee ik heb samengewerkt.

Room Y1. 167. De onderzoekerskamer. In de loop van de jaren zijn er vele bewoners geweest in onze kamer. Wouter, Johan, Jan Lucas (the general), Evert-Jan, Marije, Simone, Veera, Mr. He, Sai Uppalapati, dank jullie wel voor de gezelligheid.

(Oud)-assistenten KNO en KNO staf. De jaarlijkse ski- en zeiltrip en de vele uit de hand gelopen vrijdagmiddagborrels moeten we koesteren. Dank voor de prettige werksfeer en opleidingsklimaat.

KNO-artsen Martini ziekenhuis. Dank jullie wel voor een fantastisch perifeer jaar en de tijd die ik heb gekregen om aan mijn promotie te werken.

Naast alle mensen op het werk heb ik gelukkig ook een lieve familie en fijne vriendengroep voor de broodnodige ontspanning en relativering.

Mijn ouders, lieve pappa en mamma, dank jullie wel voor jullie liefdevolle opvoeding. Het heeft me nooit aan iets ontbroken, en ook nu voelt thuiskomen nog als een warm bad.

Anna en Zeno, dank jullie wel dat jullie ons de regelmatig de mogelijkheid geven om “uut te bloazen” in de achterhoek. Zeno, dank je wel voor de bereidheid om foto’s te maken bij mijn promotie.

Mijn zusje, Elisabeth. Volgens sommigen in de familie de slimste uit ons gezin. Jij bent vorig jaar al gepromoveerd in Rotterdam. Ik kan alleen maar hopen dat mijn promotie net zo goed gaat als die van jou. Bedankt dat je mijn paranimf wil zijn. Ik ben als oudere broer super trots op wat je allemaal hebt bereikt.

Mijn broertje, Diederik, beste Died. Jij behoort tot de niet medische minderheid in de familie, met een gezonde angst voor ziekenhuizen. Denk dat er niemand is die ik zo vaak spreek als jij. Naast dat je mijn broertje bent, ben je ook een goed maatje.

Lieve Oma, jij was de enige die mij als kind rustig kon krijgen. Inmiddels oud qua leeftijd, maar nog jong van geest. Fantastisch dat je op jouw leeftijd nog bij mijn promotie kan zijn. Ik hoop dat we nog lang van je kunnen genieten.

Beste Bas, naast paranimf ook mijn beste maatje. We kennen elkaar inmiddels al weer meer dan 20 jaar. Dank voor je vriendschap en de ondersteuning rondom de promotie.

JC Trechter. Beste Res, Hoekie, Schimmel, Brennie, Lut, Ro, Riko, Herman, Bewey en Lo. Na al die jaren doen we nog steeds veel mooie dingen met elkaar. Op naar de 3e lustrumreis in 2013. Met zijn allen!

Huize Ransdael. Veel oud-huisgenoten, weinig medici. Altijd goed om in mijn vrije tijd met jullie over iets anders te kunnen praten dan geneeskunde, iets wat medici onder elkaar toch geneigd zijn te doen. Dat we elkaar nog vaak mogen zien op huwelijken, OHD's en andere gelegenheden.

Het beste bewaren we voor het laatst.
Danna, Tijn en Fleur, mijn schatjes. Jullie relativeren alles. Ik houd van jullie!

Curriculum Vitae

CURRICULUM VITAE

Michiel Leonard Schrijvers werd op 29 juli 1978 geboren te Groningen. In 1996 behaalde hij het VWO diploma aan het Praedinius Gymnasium te Groningen. Na de middelbare school bracht hij een jaar door in Chambéry, Frankrijk, waar hij een studie Frans volgde. Vervolgens startte hij in 1997 met de studie Economie aan de Rijksuniversiteit Groningen, waarvan het propedeutisch examen werd behaald. In 1999 kon, na enkele malen uitgeloot te zijn geweest, gestart worden met de studie Geneeskunde aan de Rijksuniversiteit Groningen. Op 31 oktober 2005 behaalde hij zijn arts-examen, waarna hij op 1 november 2005 startte met het promotieonderzoek binnen de afdeling KNO-heelkunde van het Universitair Medisch Centrum Groningen. Op 1 mei 2008 werd gestart met de opleiding tot KNO-arts binnen deze afdeling onder leiding van Prof. Dr. B.F.A.M. van der Laan. Hij zal zijn opleiding afronden per 1 mei 2013.

De auteur woont samen met Danna Croonen, oogarts te Groningen, en ze hebben samen twee kinderen, Tijn en Fleur.